# Microplastics as Emerging Contaminants: Method Development, Ecotoxicity Testing and Biomonitoring in South African water Resources

Report to the
**Water Research Commission**

by

**Ntombekhaya Mgaba, Neil Griffin, Paul Kojo Mensah, Edgar Tumwesigye, Enahoro Owowenu, Zintle Mtintsilana, Chika Nnadozie & Oghenekaro Nelson Odume**

Unilever Centre for Environmental Water Quality
Institute for Water Research
Rhodes University

**WRC Report No. 2919/1/22**
**ISBN 978-0-6392-0470-3**

**July 2022**

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# EXECUTIVE SUMMARY

## BACKGROUND

The increases in the production of plastic materials globally have led to their ubiquity in the environment. To date, plastics of all sizes have been reported to be present in all type of environments, including marine, freshwater, terrestrial, agricultural lands, drinking water, and air. The ubiquity of plastic materials within the environment coupled with the fact that they are not easily degradable have raised serious concerns about the ecological and human health risk posed. Current empirical evidence suggests that the ecological and human health risk posed by plastics is influenced by several factors which include the physical and chemical properties of the plastic material, the vulnerability of the impacted biological agent, as well as concentration and distribution of plastics in the environment. In South Africa, microplastics have been reported to occur in river systems, however, not much work has been done to examine the potential toxicity and effects of microplastics on biological systems at environmentally realistic concentrations. To this end, methods for quantifying microplastics effects or their toxicity have not been well-established, although standard toxicity testing methods could be adapted. This project thus fills an important knowledge gap by investigating the occurrence and distribution of microplastics in the Swartkops and Buffalo River systems in the Eastern Cape, as well as the ecotoxicity of microplastics and plasticisers on selected test organisms at environmentally realistic concentrations.

## PROJECT AIMS

The following are the specific aims of the project

1. To convene a project inception workshop and update literature review on microplastics in freshwater environments based on national and international reports, focusing on ecotoxicity testing for human health and environmental risk assessment.
2. To develop (or adapt) methods for quantification and characterisation microplastics in freshwater systems, as well as methods for assessing the toxicity of microplastics (as chemical and physical stressors) using selected test freshwater organisms through multiple life stages.
3. To evaluate the potential toxicity of microplastics as chemical stressors due to the presence of additives (e.g. plasticisers) based on novel endpoints using selected test organisms through multiple life stages.
4. To evaluate the potential toxicity of microplastics as physical stressors, considering different shapes and sizes of microplastics particles based on novel endpoints using selected test organisms through multiple life stages.
5. To apply the developed (or adapted) toxicity-based methods for assessing the environmental risks of microplastics (as chemical and physical stressors) in selected South Africa River systems (Swartkops and Buffalo Rivers) using selected test organisms, through multiple life stages.
6. To compile a communiqué and policy document on the toxicity of microplastics in the environment as well as recommendation for microplastics monitoring in freshwater ecosystems.

## PROJECT APPROACH AND METHODOLOGY

An initial literature review, which focused on the reported effects of microplastics on freshwater organisms, from cellular to whole organism effects, was conducted. Subsequently, laboratory studies involving exposing different life stages of the freshwater snail (*Melanoides tuberculate*), shrimp (*Caridina nilotica*), zebra fish (*Danio rerio*), and tilapia fish (*Tilapia sparrmanii*) to different concentrations and types of microplastics, including polypropylene (PP) (0-6263 particles/L), polyethylene (PE) (0-3691 particles/L) and polyvinyl chloride (PVC) (0-2880 particles/L) were conducted. For PP, fibres constitute 30% of the particles, spheres 20% and irregularly shaped particles constitute 50% of the test solution. For PE, 65% of the particles in solution were fibres, 15% were sphere and 20% were irregularly shaped. For the PVC, 20% of the particles were fibres, 30% spheres and 50% irregularly shaped. The average sizes of the particles used for the experiments were 18.4 µm for PP, 29.3 µm for PE and 24.8 µm for PVV. The size ranges were 1.7-1400 µm for PP, 10-2500 µm

for PE, and 2.9-1600 µm for PVC. Various endpoints were tested including biochemical effects, growth, and reproductions. Test organisms were also exposed to selected plasticisers, including Bisphenol A, Dibutyl Phthalate, and calcium stearate at environmentally realistic concentrations. *Melanoides tuberculate*, *Caridina nilotica*, *Danio rerio* and *Tilapia sparrmanii* were exposed to 0-0.00215 µg/l of dibutyl phthalate, 0-4 µg/l of Bisphenol A and 0-5 mg/l calcium stearate. To determine the presence and prevalence of microplastics in freshwater, water samples were periodically collected from the Swartkops and Buffalo Rivers over one hydrological year, taking samples from distinct biotopes.

## RESULTS AND DISCUSSION

### Current science of the effects of microplastics on environmental health

A review of current literature indicated that microplastics effects have been reported at the cellular, tissues and whole organism levels, but concentrations at which effects are observed are usually higher than those generally reported in the field. According to literature, cellular and biochemical effects observed are mostly related to changes in gene expression, effects on enzymatic activity and oxidative stress. At the tissue level, blockage of the gut, translocation of microplastics to the liver and abrasion of the gut are some of the typical effects reported. Whole organism effects are mainly behavioural and relate mostly to feeding behaviour. Observed effects are mediated by the test organisms, concentrations of microplastics, shapes and sizes of microplastics, and exposure duration. With regards to human health, inhalation and ingestion via drinking water and food materials are the main exposure routes, in addition to high-risk occupations. Inflammatory responses, and lesions, production of reactive oxygen species (ROS) and genotoxicity effects have been reported for humans in the literature.

### Laboratory studies on the effects of microplastics on aquatic organisms

In terms of the laboratory experiments conducted in this study, significant effects owing to exposure to microplastics were not observed for most of the test organisms at the test microplastic concentrations, and particle sizes and shapes investigated. In the results obtained using freshwater snail (*Melanoides tuberculate*), no significant effects were observed for adult snail reproduction and growth. Similarly, the juvenile snail did not exhibit any effects due to exposure to microplastics. However, the dose-response curve suggested that growth is slowed at higher concentrations in young snails to a greater extent than in adult snails. There was high variations in the data, and the fitted curve could not be said to show that polyvinyl chloride response significantly. For Tilapia, no effects on their growth at the test concentrations were observed after 21 days of exposure. Thus, using the microplastics at the concentrations tested, no significant toxicological responses were observed, except for the effects of polypropylene particles on fish growth. However, it was observed that fish significantly egested consumed microplastics, through a process of gut clearing. It needs to be noted that even though no responses were found during the exposure tests, this does not rule out other potential impacts owing to, for example, long term plastic accumulation in the gut and consequent feeding reduction, which were however not observed in the present study.

### Laboratory studies on the effects of plasticisers on aquatic organisms

Apart from the effects of Bisphenol A on snail reproduction, minimal effects due to exposure to plasticisers were observed for most of the test organisms investigated in this study. Bisphenol A had a profound effect on the reproduction of *M. tuberculate*, significantly decreasing the production of offspring and overall reproductive success at the test concentrations. The other plasticisers had no clear effect on snail reproductive success. This result indicates that plastics with a Bisphenol A could pose a potential significant ecological risk in the environment if the plasticiser is leached. At higher concentrations of the plasticisers, often at concentrations higher than those reported in the environment, effects on the test organisms could be detected.

### Monitoring the presence of microplastics in the Swartkops and Buffalo Rivers

The results of the field study indicate widespread occurrence of microplastics, providing further empirical evidence to the claim of ubiquity of microplastics in South African freshwater systems. This observation raises the question of what such high levels of microplastics might mean for river biota. In the microplastic toxicology

exposures undertaken in this project, these field concentrations were relatively low, and in laboratory exposures, no toxic impact was detected on a range of endpoints. These results accord with international observations that microplastic exposures that elicit a biotic response are often at levels of microplastics far higher than are encountered in the environment. In a similar light, European scientific advisers concluded that no known risks were posed by environmental levels of microplastics. It is, therefore, possible that the physical effect of this level of microplastic particles on biota in the environment may be limited or undetectable.

**CONCLUSION**

The findings of this study suggest that, generally, plasticisers seem to impact on stress enzyme activity, though many other endpoints showed little response. Given the lack of significant response of most endpoints to the plasticisers in other tests, and the near absence of clear responses to physical exposure to microplastics, it is of value to identify one pathway that may lead to microplastic impacts in the environment. Importantly, study has contributed to providing research-based tools that can be used to protect freshwater resources from microplastic pollution in South Africa. Techniques such as characterisation of microplastics and toxicity tests for microplastics using different aquatic organisms that are useful for investigating microplastics impact in the aquatic environment have been explored as important outcomes of this project. As pointed out in the literature, most methods microplastics research were developed for the marine environment. In this study, these methods were adapted, modified, and/or optimised to develop new methods to enhance their applications to studying microplastics in freshwater ecosystems with a focus on freshwater systems in South Africa. In view of this, methods for undertaking biomonitoring studies involving microplastics in freshwater systems and ecotoxicological studies using freshwater organisms have been developed as outcomes of this project.

**RECOMMENDATIONS FOR POLICY AND IMPLEMENTATION**

- Although the results of the present study seem to suggest that microplastics posed minimal ecological risk, at least to the biota tested at concentrations reported in the environment, policy instruments should be directed towards minimizing the entry of plastics materials into freshwater resources. Both punitive- and incentive-based systems can be implemented to prevent and/or minimize plastic pollution of freshwater systems. Such policy instruments should consider the plastic value and production chains, as well as behavioural and technological driver of change towards plastic reduction in the environment.

- Despite the empirical evidence suggesting the presence of microplastics in South African riverine systems, their occurrence and distribution as well as potential toxicity on indigenous species are poorly studied. A multidisciplinary microplastic monitoring network and programme is recommended. Such a programme would seek to generate data on their occurrence, geospatial distribution, use, toxicity, and human and ecological risk. Such data would be critical for evidence-based policy instruments in South Africa.

- Policy instruments should target both hard and soft measures, such as behavioural change, social learning, and technological innovations for the recycling, re-use, and reduction of plastic materials through the principles of circular and green economy.

**RECOMMENDATIONS FOR FUTURE RESEARCH**

The following are recommended for future studies

- An ecological functional approach to toxicity study of microplastic is recommended. This could include an analysis of the effects of microplastics on feeding efficiency, feeding behaviour, oxygen uptake and metabolic function. It is likely that effects could be observed if a functional approach is followed.

- Microplastics occurrence and distribution in the riverine systems is potentially mediated by hydrology, hydraulics characteristics and microplastic movements (lateral, vertical and horizontal). A mechanistic approach that seeks to understand the influences of hydrology, and hydraulics on the distribution of microplastics, and thus, the potential exposure of riverine organisms is recommended.

# ACKNOWLEDGEMENTS

The project team wishes to thank the following people for their contributions to the project:

| Reference Group | Affiliation |
|---|---|
| Pamela Nxumalo | Department of Environment, Forestry and Fisheries |
| Nonhlanhla Kalebaila | Water Research Commission (Chair) |
| Henk Bouwman | North-West University |
| Tam Msagati | University of South Africa |
| Bridget Shaddock | Golder Associates Research Laboratory |
| David Odusanya | Department of Water and Sanitation |
| Linda Godfrey | Council for Scientific and Industrial Research |
| Abraham Addo-Bediako | University of Limpopo |
| Ntuthuko Masikane | University of Zululand |
| Peter Manyara | International Union for Conservation of Nature |
| Stanley Nzama | Department of Water and Sanitation |
| Byron Grant | Ecology International |
| Annabé Pretorius | Plastics SA |
| Randal Albertus | Sasol |
| Carina Verster | North-West University |

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# ACRONYMS & ABBREVIATIONS

| | |
|---|---|
| AChE | Acetylcholinesterase |
| ATR-FTIR | Attenuated total reflection Fourier transform infra-red spectroscopy |
| AVANI | All-purpose Velocity Accelerated Net Instrument |
| BPA | Bisphenol A |
| CAS | Calcium stearate |
| CDC | Centre for Disease Control and Prevention |
| DBP | Dibutyl phthalate |
| DEEEP | Direct estimation of ecological effect potential |
| DEHP | Di-2-ethylhexyl phthalate |
| DEHP | di-2-Ethylhexyl phthalate |
| DNA | Deoxyribose nucleic acid |
| DWA | Department of Water Affairs |
| DWAF | Department of Water Affairs and Forestry |
| DWS | Department of Water and Sanitation |
| EC | Electrical conductivity |
| EC10 | Effect concentration 10% |
| EC50 | Effect concentration 50% |
| ECHA | European Chemicals Agency |
| ECx | Effect concentration x% |
| EDTA | Ethylenediaminetetraacetic acid |
| EFSA | European Food Safety Authority |
| EU | European Union |
| FTIR | Fourier transform infrared spectroscopy |
| GC/MS | Gas chromatography/mass spectrometry |
| GESAMP | Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection |
| HDPE | High density polyethylene |
| HDPE | high-density polyethylene |
| HSP | heat shock proteins |
| IDH | Isocitrate dehydrogenase |
| IDH | isocitrate dehydrogenase |
| IWR | Institute for Water Research |
| LDH | Lactate dehydrogenase |
| LDH | lactate dehydrogenase |
| LDPE | low-density polyethylene |
| LPO | Lipid oxidation |
| LPx | Lipid peroxidase |
| NCBI | National Centre for Biotechnology Information |
| NIOSH | National Institute for Occupational Safety and Health |
| NOAA | National Oceanic and Atmospheric Administration |
| PAH | Polycyclic aromatic hydrocarbon |
| PAHs | polycyclic aromatic hydrocarbons |
| PCB | Polychlorinated biphenyl |
| PCBs | polychlorinated biphenyls |
| PE | Polyethylene |

| | |
|---|---|
| POPs | persistent organic pollutants |
| PP | Polypropylene |
| PVC | Polyvinyl chloride |
| SAPEA | Science Advice for Policy by European Academies |
| SEM | Scanning electron microscopy |
| UCEWQ | Unilever Centre for Environmental Water Quality |
| UDP | Uridine diphosphate |
| UGD | UDP-glucoronate decarboxylase |
| UGE | UDP-glucose 4-epimerase |
| UN | United Nations |
| UNEP | United Nations Environmental Programme |
| WHO | World Health Organisation |
| WRC | Water Research Commission |

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# CHAPTER 1: BACKGROUND

## 1.1 INTRODUCTION

Plastic production has grown significantly in the past few years, with an annual production estimated at 322-380 million metric tons by 2015, a significant leap from the 1.7 million metric tons estimated in 1950 (Geyer *et al*., 2017). Plastic production and use thus represent one of the many advances in human technological development, with about 40% of plastics produced used chiefly for packaging (Geyer *et al*., 2017). It is estimated that more than 8300 million metric tons of plastics have been produced to date, and about 60% of these plastics end up in the environment where they accumulate in large quantities (Merga *et al*., 2020). The accumulation of plastics in the environment has seen growing concern from scientists, natural resource managers, policymakers and the general public, who are worried about potential threats to human health and integrity of the environment in which they accumulate (Adam *et al*., 2020)(Horton and Dixon, 2017).

Accumulation of plastic materials in the environment occur in different sizes and shapes and consist of various polymers of varying densities (Ivleva *et al*., 2017). Plastics manufactured in size range ≤ 5 mm are usually considered primary microplastics, typically in small pellets, glitter and microbeads (Horton and Dixon, 2017). Secondary microplastics are small plastic materials resulting from the breakdown processes of plastics in the environment and usually occur in fibres, fragments and films (Horton and Dixon, 2017). Mechanisms responsible for the breakdown of plastics in the environment include mechanical degradation, e.g. road wear, tyre abrasion, physical weathering and washing of textile materials (Hermandez *et al*., 2017), as well as chemical breakdown processes and UV-induced breakdowns. Biological degradation has been reported, with organisms capable of ingesting and shredding plastic materials, e.g. waxworm (Yang *et al*., 2014). Thus, when microplastics accumulate in the environment, especially freshwater ecosystems, they can cause ecological impairment of rivers and streams (Windsor *et al*., 2018).

Microplastics may enter into freshwater environments through inadequately treated wastewater effluent, urban stormwater return flow, run-off from informal settlements and agricultural farmlands (Ivleva *et al*., 2017). More extensive plastic materials in the landfill and those indiscriminately disposed of may enter into freshwater environments, where they become broken down into microplastics. Thus, freshwater systems represent a complex environment for microplastics since they directly receive microplastics from the terrestrial environment, serving as a primary link between terrestrial and marine environment through microplastic transport and aiding the breakdown of breakdown microplastics further breakdown of microplastics into nano plastics (Merga *et al*., 2020). Further, freshwater sediments act as a sink for microplastics, where toxic chemicals may become adsorbed onto their surfaces while forming plastics for potentially pathogenic microbes, all of which become transported to the marine environment (Ivleva *et al*., 2017). However, microplastics' occurrence, ecology, and ecotoxicity in freshwater environments are underexplored in Africa, compared to the marine environment.

In South Africa, microplastics have been reported widely in freshwater environments (Lambert and Wagner, 2018), with little emphasis on their toxicity and ecology. In light of this, the project team organised a one day workshop on 25 June 2019. The workshop attracted delegates from academia, the plastic industry, the water sector practitioners and the military. After exploring the project topic with all representatives, it became clear that in South Africa, very little is known about the extent of the threat posed by microplastics.

Therefore, the question of what threats are posed by microplastics in aquatic environment arose. This question thus guides the analytical review presented in this report. Given that microplastics can be characterised in terms of i) sizes ii) shape, iii) polymer makeup, iv) plasticisers and additive composition v) rate and degree of degradation, vi) uptake as well as vi) being vectors for potentially toxic chemicals and microbes, the review presented searches for empirical evidence of microplastic impact attributable to any of how microplastics can be characterised. The intention is that once the microplastic characteristics that pose the greatest challenge to the environment are identified, the management and mitigation strategies can thus be better directed.

## 1.2 PROJECT AIMS

Therefore, in responding to the identified gaps in South Africa, in terms of microplastics toxicity, occurrence and effects in freshwater systems, the following are the main objectives of the project.

1. To convene a project inception workshop and update literature review on microplastics in freshwater environments based on national and international reports, focusing on ecotoxicity testing for human health and environmental risk assessment.
2. To develop (or adapt) methods for microplastics quantification and characterisation, as well as methods for assessing the toxicity of microplastics (as chemical and physical stressors) using selected test organisms through multiple life stages.
3. To evaluate the potential toxicity of microplastics as chemical stressors due to the presence of additive (e.g. plasticisers) based on novel endpoints using selected test organism through multiple life stages.
4. To evaluate the potential toxicity of microplastics as physical stressors, considering different shapes and sizes of microplastics particles based on novel endpoints using selected test organisms, through multiple life stages.
5. To apply the developed (or adapted) toxicity-based methods for assessing the environmental risks of microplastics (as chemical and physical stressors) in selected South Africa River systems (Swartkops and Buffalo Rivers) using selected test organisms, through multiple life stages.
6. To compile a communique and policy document on the toxicity of microplastics in the environment as well as recommendation for microplastics monitoring in freshwater ecosystems.

## 1.3 SCOPE AND LIMITATIONS

The project comprised both laboratory and field studies to assess the impacts of microplastics on freshwater systems using ecotoxicology and biomonitoring approaches. Different microplastic polymers of various shapes and sizes, as well as different plasticisers, were used as physical and chemical stressors, respectively. Efforts were made to ensure that physical and chemical stressors used were of environmentally realistic levels, i.e. concentration levels often reported in the literature as found in the natural environment. In this study, the short-term exposure time was taken to be not exceeding 4 days, while long-term was taken to be not less than 15 days. Although these exposure times could be acceptable and appropriate for chemical stressors (plasticisers), the same cannot be said for the physical stressors (microplastic polymers). This, therefore, adds to the uncertainty in predicting impacts of microplastics in the natural environment. Furthermore, ATR-FTIR was used to characterise colour, chemical composition, size, shape, morphology, and structure of the polymer because its non-invasive nature ensures that it did not change or destroy the sample during analysis.

Scanning Electron Microscopy, which can identify inorganic plastics additives and give information about the shape and size of the particles (Hanvey et al., 2017), and Gas Chromatography (GC) coupled to Mass Spectroscopy (MS) for identifying polymer typology by obtaining structural information about macromolecules were not used. These were some limitations of the project.

# CHAPTER 2: MICROPLASTICS AND THEIR EFFECTS ON FRESHWATER AQUATIC ORGANISMS AND HUMAN HEALTH – A REVIEW

## 2.1 INTRODUCTION

This chapter presents a review of current literature on microplastics as emerging contaminants of concern. Much has been written on the accumulation of microplastics within biota and the environment. The majority of microplastic particles entering freshwater, and ultimately accumulating in biota, are primarily from secondary microplastics generated by the breakdown of larger plastic materials, for example, single-use packaging, tyres, fibres from synthetic fabrics, and road paint particles (Horton *et al*., 2017a). These microplastics enter freshwater either through surface and agricultural runoffs or direct disposal of wastes due to poor waste management (Free *et al*., 2014). Effluent discharges from wastewater and sewage treatment plants have been identified as a potentially significant point source of microplastics that contribute to the entry of microplastics into freshwater (Cole *et al*., 2011). The impacts of these discharges into the aquatic environment are not always clearly understood.

Experts advising the European Commission reviewed the evidence of microplastic and nanoplastic impacts and found several examples of impacts in controlled exposures (SAPEA, 2019). However, they note that tests often used only microbeads, but not microfibers, in experiments, and that impacts were often found at plastic levels unrealistic in the natural environment. Other authors have noted the environmentally unrealistic levels of microplastic exposure in many exposure assessments (Triebskorn *et al*., 2019; GESAMP, 2016; Tang, 2017; Lenz *et al*., 2016). Exposure times were often short, and these factors add to uncertainty in predicting the impacts of plastics in the natural environment.

## 2.2 MICROPLASTICS AS EMERGING CONTAMINANTS OF CONCERN

### 2.2.1 Overview

The toxicity of microplastics is linked to the fact that they contain additives and can also act as co-pollutants. Plastic may contain different additives that can affect an organism differently, such as behavioural change, reproduction inhibition or increased mortality. Aquatic organisms have been exposed to both additives and microplastics in different studies, including Chen *et al*. (2015), Zhu *et al*. (2016), Lambert *et al*. (2017) and Gobas *et al*. (2017). Different plastic additives such as phthalates and Bisphenol-A have been used for ecotoxicological studies. These additives are mainly representatives of PE (Bisphenol A) and PVC (phthalate) (Table 2-1). Some results indicated that sperm quality and eggs reproduction of fish decreased after being exposed to phthalate (Zhu *et al*., 2016). Microplastics can act as vectors for other pollutants when used, for example, in consumer and pharmaceutical products as well as medical and industrial applications. They can also come into contact with other chemical substances such as preservatives, surfactants and active ingredients. Microplastic particles may also come into contact with contaminants in the environment through improper disposal in wastewater and landfill leachates. These interactions with other chemical compounds before, during and after their intended use ensure that the hitherto inert and non-toxic microplastic particles become potential vectors of toxic compounds (Rist and Hartmann, 2018). Microplastics may also act as carriers

for hydrophobic organic chemicals, which have low water solubility. Adsorption to microplastics enables them to become more mobile, increasing their transport and consequently their distribution and bioavailability (Teuten *et al*., 2007).

**Table 2.1: Common types of microplastics and typical additives**

| Plastic polymer | Abbreviation | Additives | Hazardous substances |
|---|---|---|---|
| Polyethylene | PE | Antioxidant<br>Flame retardant (cable insulation and electronic application) | Bisphenol A |
| Polyvinyl chloride | PVC | Plasticiser<br>stabilizer | Phthalate |
| Polypropylene | PP | Plasticiser | Calcium stearate |

### 2.2.2 Effects of microplastics on aquatic organisms

The presence of microplastics within the marine environment is well known and have received the most attention. Likewise, threats posed by marine microplastics has been recognised for some time (Gomiero *et al*., 2019; UNEP, 2016). Scientific reports on the threat posed by microplastics date back as far as the 1970s (UNEP, 2016), while records of plastic entanglement with equipment date back to the 1950s (Ostle *et al*., 2019). Sources of marine microplastics include land-based sources such as single-use plastics (including packaging, household goods, consumer goods and sanitary items) and multiple-use plastics (including electronics and domestic, commercial and industrial goods), as well as marine sources such as fishing, aquaculture, shipping and recreation (UNEP, 2016). Impacts on marine biota are largely through entanglement and ingestion, with indirect effects to habitat damage (UNEP, 2016).

Recently, an increasing number of studies have also reported presence of microplastics in freshwater, aerial, and terrestrial systems (Dris *et al*., 2016; de Souza Machado *et al*., 2018; Eerkes-Medrano *et al*., 2015). With an estimated plastic production of 550 million tons in 2018, the leakage of plastic materials into the environment is expected to increase (Gomiero *et al*., 2019). Recycling plastics is possible, though most of this is mechanical rather than by depolymerisation or other methods (Garcia and Robertson, 2017). Despite efforts made to promote plastics recycling, only 9% of plastic produced has been recycled, while 12% has been incinerated, and the remainder is in landfills and the environment (Geyer *et al*., 2017). None of the commonly used plastics is biodegradable, resulting in an ongoing accumulation of plastics in different environments.

Hazards and toxicity effects posed by exposure to microplastics may result from several microplastic characteristics (SAPEA, 2019 and references therein, Triebskorn *et al*., 2019 and references therein). They may be caused by the physical impact of microplastic particles, obstruction of feeding, chemical toxicity of plastic components, chemical toxicity of compounds adsorbed to the microplastic particles, and interactions between any of these factors. As a further complication, organisms are exposed to a range of plastic types, particle sizes, and shapes (SAPEA, 2019; Triebskorn *et al*., 2019). Microplastic impacts may affect organisms at various levels of organisation (from cellular, tissue, organ and to the complete organism). This review will concentrate on the known effects of microplastics, with or without adsorbed compounds, giving specificity where appropriate.

#### *2.2.2.1 Molecular and cellular impacts*

Microplastics have been found to change DNA in several ways. For example, microplastic uptake by *Mytilus galloprovincialis* led to changes in gene expression (among other impacts) in the mussels (Avio *et al*., 2015). This action was caused by the adsorption of pyrene onto plastic beads and was not an apparent response to a plastic polymer itself. In a similar way, compounds adsorbed to low-density polyethylene (LDPE) led to DNA strand breakage in the clam *Scrobicularia plana* (O'Donovan *et al*., 2018). Polystyrene particles had the same effect (Ribiero *et al*., 2017). Changes in gene expression were also found in adult and juvenile *Daphnia magna* exposed to a mix of polymers without adsorbed compounds (Imhof *et al*., 2017). It is worth noting that these taxa are filter feeders and so are likely to have a high rate of microplastic ingestion. However, the microalga *Chlamydomonas reinhartdi* also showed changed gene expression patterns after long exposure to polypropylene (PP) and high-density polyethylene (HDPE) (Lagarde *et al*., 2016). The majority of microplastic effects at the DNA level reflect altered gene expression as a result of the polymer itself or adsorbed compounds, but direct gene damage was also reported.

Another microplastic-related DNA change is the colonisation of microplastics by bacteria to form plastisphere, which is a part of the global ecosystem based on plastics, especially one consisting of floating plastic debris and the microbes and other organisms that live on it. Antibiotic resistance genes and metal resistance genes have been found in greater numbers and diversity in the plastisphere than the surrounding environment (Yang *et al*., 2019). This may, in part, result from altered diversity and metabolic function in biofilms making up the plastisphere (Miao *et al*., 2019). However, it has been found that microplastic colonisation increases plastid and gene exchange between bacteria (Arias-Andres *et al*., 2018). Changes to cell function have been found across a range of taxa in response to microplastic exposure or ingestion and a result of other compounds adsorbed to the microplastics.

One mechanism for this that is linked to DNA and DNA regulation is the production of various protective proteins or peptides when exposed to microplastics. An example of these includes heat shock proteins (HSP), discovered in Drosophila exposed to heat stress but have since been found in all living things and are produced in response to a range of stresses (Moseley, 2000). Another example is antioxidant proteins and peptides, which neutralise free radicals to maintain the cellular oxidation/antioxidation balance (Zhang *et al*., 2016; Feng *et al*., 2017). Both have been found to respond to microplastic exposure. Other enzymes, for example, acetylcholinesterase, have also been found to respond to microplastic exposure and ingestion. Microplastic-induced stresses to animals' antioxidative systems, energy metabolism and nervous systems are also reviewed in Prokíc *et al*. (2019).

*Chlamydomonas reinhardtii cultures* exposed to PP and HDPE showed increased expression of genes involved in sugar biosynthesis, in particular UDP-glucoronate decarboxylase (UGD) and UDP-glucose 4-epimerase (UGE), while expression of other sugar biosynthetic genes was unchanged or changes were insignificant (Lagarde *et al*., 2016). Gene expression in a range of other microalgae was also affected by microplastic exposure (Yokoto *et al*., 2017 and refs therein, Prata *et al*., 2019 and refs therein). *Daphnia magna* exposed to microplastic mixes showed changes in the expression of several, though not all, genes assessed (Imhof *et al*., 2017). Responses that changed included several HSPs. Oxidative damage was found at varying levels in different tissues in the clam Scrobicularia plana exposed to microplastics, and changes matched this in the activity of several different enzymes (O'Donovan *et al*., 2018).

Oxidative damage and neurotoxicity were found in Scrobicularia plana exposed to polystyrene particle (though at concentrations that exceed the natural state) (Ribiero *et al*., 2017). Another filter feeder, *Mytilus galloprovincialis*, showed stress protein increases when exposed to microplastics with adsorbed

compounds (Avio *et al*., 2015). Other authors have also found stress protein expression in *M. galloprovincialis* in response to microplastic exposure (Détrée and Gallardo-Escárate, 2018). Barboza *et al*. (2018a) found that microplastics exposure led to acetylcholinesterase (AChE) inhibition, increased lipid oxidation (LPO) in the brain and muscle, and changed the activities of lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) in the seabass *Dicentrarchus labrax*. Intriguingly, microplastic exposure also altered patterns of mercury bioaccumulation in this fish. PVC exposure altered stress protein expression in the seabream *Sparus aurata* (Espinoza *et al*., 2017).

The impacts of microplastics on algal photosynthesis and growth is contested. Sjollema *et al*. (2016) report that algal (*Thalassiosira pseudonana* and *Dunaliella tertiolecta*) photosynthesis did not change, but that growth was inhibited. Bhattacharya *et al*. (2010) found that photosynthesis in Chlorella sp. and Scenedesmus sp. was inhibited by microplastic exposure and that increased reactive oxygen species were produced in the process. They also found that test taxa adhered to microplastics, a finding confirmed by Lagarde *et al*. (2016), who observed that aggregation of *Chlamydomonas reinhardtii* depended on polymer type and was accompanied by stress protein production. Lagarde *et al*. (2016) noted that clumping of algae and microplastics led to increased sinking and transport of aggregates to the sediment.

Decreases in chlorophyll levels in algae exposed to microplastics have been recorded, an effect which was not attributed to the shading of the algae (Besselling *et al*., 2014; Zhang *et al*., 2017). However, Prata *et al*. (2019, and references therein) reviewed the effect of microplastics on algae, including effects due to nutrient depletion and osmotic change, and concluded that the impact of microplastics on algae at environmentally realistic levels is likely to be minimal and for the most part temporary. They also noted that this conclusion has been reached on the basis of scarce evidence and that there is a need for further research to clarify the impact of microplastics on algae. They also indicated the potential for impacts on algae to arise from direct impacts on grazers reducing pressure on algal populations. Burton (2017), writing with reference to microbeads, also proposes that impacts at environmentally realistic levels are likely to be minimal.

#### *2.2.2.2 Tissue and organ impacts*

It is broadly accepted that microplastics have negative impacts on biota. However, owing to the capacity of microplastics to adsorb other compounds onto the microplastic surface, it is desirable that the cause of impacts be identified as native, or virgin, microplastic itself or adsorbed compounds or both. Compounds that have been found include antibiotics (Li *et al*., 2018), heavy metals (Brennecke *et al*., 2016), and a range of organic compounds, some of which are toxic or have significant ecological risk profiles (Cole *et al*., 2011;, Avio *et al*., 2017; Wang *et al*., 2018). The impact of uncontaminated microplastics may be contested. For example, van Moos *et al*. (2012) found a strong inflammatory response to ingested microplastics in the digestive gland of Mytilus edulis (and, in different fish taxa, Lu *et al*. (2016) and Ding *et al*. (2018) also found clear microplastic impacts), while Jovanovíc *et al*. (2018) found no clear evidence of impacts in gilt-head bream (*Sparus aurata*). These studies are not directly comparable as different test taxa were used, and different microplastics were assessed.

Nevertheless, the comparison illustrates that responses are likely to vary between taxa and polymer types. The majority of studies reviewed assessed larger organisms and fish in particular. A substantial body of work on various bivalves was included. Research on tissue or organ level impacts was rare, except for bivalves. Research on single-celled taxa, which do not contain tissues or organs, is reviewed under cell impacts above.

Zhao *et al*. (2017) found increased intestinal permeability owing to PS nanoparticles in the nematode *Caenorhabditis elegans*. Lei *et al*. (2018) also found increased intestinal damage and leaking in *C. elegans* and also in the zebrafish *Dania rerio*. Wang *et al*. (2019) found changes to the intestinal epithelium that included oil droplet accumulation and disordering of microvilli in the brine shrimp *Artemia parthenogenetica*. Peda *et al*. (2016) found intestinal damage in European sea bass *Dicentrarchus labrax* resulting from microplastic exposure. On the other hand, Jovanovíc *et al*. (2018) found that uncontaminated microplastics caused no significant damage to the intestine or gut of gilt-head seabream *Sparus aurata*, following exposure, largely left the gut. Cole and Galloway (2015) found microplastic ingestion without impact by larval Pacific oyster Crassostrea gigas (although no specific histological assessment was undertaken).

Likewise, Ašmonaitė *et al*. (2018) found no measurable intestinal stresses in Rainbow trout *Oncorhynchus mykiss* owing to microplastics. It seems, therefore, that intestinal and gut damage may occur but is not always present, and the impact of different microplastics on the gut of consumers' needs further research to determine the impacts of particular microplastic polymer, size and shape. The intestine and gut are most exposed to ingested microplastics and, as a result, are logical sites for microplastic impact. This may increase in future, despite the generalisation that microplastic presence in the gut is ephemeral and particle retention in it is low (Jovanovíc, 2017).

Many authors have noted the potential for gut blockage owing to microplastics (e.g. Jovanovíc, 2017; Galloway and Lewis, 2016; Mizraji *et al*., 2017), though there is little data on this phenomenon, and its importance is unknown. However, microplastic-induced changes in feeding patterns have been recorded. For example, uptake or translocation of microplastics by various fish is noted above. Once the microplastics have entered the tissue of a fish, they are commonly translocated to the liver (Jovanovíc, 2017; Lu *et al*., 2016; Abbasi *et al*., 2018). Microplastics can translocate to other tissues, too (Abbasi *et al*., 2018). The translocation of microplastic particles in this way is often size-specific (Lu *et al*., 2016), and as a result, experiments using particles of a fixed size might not detect meaningful levels of translocation. Microplastics in fish livers are commonly associated with markers or indicators of oxidative stress (Ding *et al*., 2018; Espinosa *et al*., 2017; Karami *et al*., 2016; Lu *et al*., 2016; Rochman *et al*., 2017). In juvenile mitten crabs *Eriocheir sinensis*, stress levels increase in the hepatopancreas (Yu *et al*., 2018). However, studies found no impact of microplastics, particularly uncontaminated or virgin microplastics, in this way (Jovanovíc *et al*., 2018; Ranieri *et al*., 2018).

Microplastics have been found to accumulate in other taxa and in other organs and are likewise associated with stress markers. However, several types, sizes, and shapes of microplastics might elicit different responses, and all taxa do not respond the same way. An obvious point of exposure for aquatic fauna is the gill surface, and microplastics have been found to attach to and be taken up at the gill, which commonly leads to stress. Research has found microplastic impacts in the gills of fish (Abbasi *et al*., 2018; Barboza *et al*., 2018a; Espinosa *et al*., 2017; Lu *et al*., 2016), crabs (Yu *et al*., 2018; Watts *et al*., 2014) and bivalves (Avio *et al*., 2017; Détrée and Gallardo-Escárate, 2018; Ding *et al*., 2018; Guilhermino *et al*., 2018; Kolandhasamy *et al*., 2018; O'Donovan *et al*., 2018; Paul-Pont *et al*., 2016; Ribiero *et al*., 2017; van Moos, 2012). Impacts on fish brains have been recorded, impacting acetylcholinesterase activity (Ding *et al*., 2018; Barboza *et al*., 2018a). Microplastics have also been found in other tissues and haemolymph (Kolandhasamy *et al*., 2018; O'Donovan *et al*., 2018).

#### *2.2.2.3 Whole organism impacts*

Given the impacts linked to microplastics at the cellular and at the tissue level, it is not surprising that whole organism impacts have also been found. Some may relate directly to cellular or tissue impacts, but other impacts may result from other physical or chemical properties. Jovanovíc *et al*. (2017) note

the change to behaviour that has been observed. As microplastics are taken up into the gut in many taxa, changes in feeding behaviour are to be expected. Cole *et al*. (2013) exposed a range of zooplankton to microplastics consumed and later egested. Consumption of microplastics and algae decreased algal feeding in the copepod *Centropages typicus,* which would impact organism energetics. Microplastic accumulation led to feeding inhibition in the bivalve *Corbicula fluminea* (Guilhermino *et al*., 2018). Feeding inhibition was also noted in the brine shrimp *Artemia parthenogenetica* (Wang *et al*., 2019). Crucian Carp (*Carassius carassius*) exposed to nanoparticles altered their feeding behaviour by being less active, increasing feeding time, staying closer together, and being less explorative (Mattson *et al*., 2018). Feeding in jacopever (*Sebastes schlegelii*) was depressed by microplastics due to several feeding and foraging activities (Yin *et al.,* 2019). Microplastics also reduced feeding in Hydra attenuate (Murphy and Quinn, 2018). Unusually, virgin microplastics (as opposed to those that had developed a microbial biofilm) were found to be preferentially fed on by a hard coral, which led to some retention of microplastics (Allen *et al*., 2017).

Microplastic exposure has been found to depress reproduction rates in a wide range of taxa. Murphy and Quinn (2018) found Hydra attenuate reproduction was decreased in the presence of large amounts of microplastics. Besseling *et al*. (2014) found that exposure to microplastics decreased growth in the green alga *Scenedesmus obliquus* and depressed reproduction in *Daphnia magna* by decreasing the count and size of neonates while increasing the number of malformations in the neonates. Reproduction in *Ceriodaphnia dubia* was also depressed following exposure to microplastics, though only at high levels (Ziajahromi *et al*., 2017). Oyster reproduction was decreased in the presence of microplastics through decreased oocyte number and diameter, and lower sperm velocity, leading to decreased larval yield as well as lower larval development (Sussarellu *at al*., 2016).

Other changes in behaviour that may lead to impacts on community resilience are found as a result of microplastic contamination. The European seabass (*D. labrax*) showed decreased swimming velocity and resistance time in the presence of microplastics and exhibited lethargic and erratic swimming behaviour (Barboza *et al*., 2018b). In jacopever (*Sebastes schlegelii*), microplastic contamination led to weakened feeding activity and exhibited reduced swimming speed, and range of movement (Yin *et al*., 2018). In crucian carp (*Carassius carassius*), nanoplastic exposure led to increased feeding time, a tendency to stay closer together than normal, and being less explorative, together resulting in reduced feeding activity (Mattson *et al*., 2015). In zebrafish Danio rerio nanoplastic exposure led to reduced larval size and locomotion, a pattern that was exacerbated in the presence of α-ethynylestradiol (Chen *et al*., 2017). In a study comparing the impact of microplastics with different shapes, Choi *et al*. (2018) found that irregular-shaped microplastics decreased swimming behaviour by reducing the total distance travelled and the maximum velocity of sheepshead minnow (*Cyprinodon variegatus*), but no change was apparent when spherical particles were tested.

### 2.2.3 Effects of microplastic on human health

#### *2.2.3.1 Exposure*

Microplastics are available to humans via various pathways (WHO, 2019; Lehner *et al*., 2019; Cox *et al*., 2019). Rates of microplastic ingestion via these pathways may be quantified but may also be ill-understood and under-researched (WHO, 2019). In this light, calls have been made to ensure that research and statements about microplastic exposure and health impacts on humans be made in terms of environmentally realistic levels of microplastics (Rist *et al*., 2018; Wright and Kelly, 2017). Microplastics may be ingested in drinking water (WHO, 2019; Kosuth *et al*., 2018; Cox *et al*., 2019). There are several sources of these microplastics, and accounting for these is notoriously challenging

(SAPEA, 2018; WHO, 2019). Notable sources include cosmetics (for microbeads), synthetic fibre production and cleaning (for microfibers), plastic waste, city dust and tyre abrasion (SAPEA, 2019 and references therein). These reach freshwater via several routes. These include run-off from land-based sources, wastewater effluents, mishandled plastic wastes and agricultural practices (Alimi *et al*., 2018; WHO, 2019 and references therein). Different water sources have been found to have differing loads of microplastics (Kosuth *et al*., 2018; Cox *et al*., 2019; Schymanski *et al*., 2018). Despite initial expectations, no clear difference between glass and plastic bottles with respect to microplastic contents was found (Kosuth *et al*., 2018; Schymanski *et al*., 2018; Cox *et al*., 2019 and references therein).

Cox *et al*. (2019), using data available at the time of writing, concluded that bottled water had more microplastics than tap water. Still, an inspection of the data underlying their analysis reveals much variation in plastic content of bottled water, and somewhat less for tap water. Cox *et al*. (2019) modelled human consumption of microplastics. They concluded that microplastic consumption owing to bottled water was higher than that from tap water, although bottled water microplastic content was very variable. Koelmans *et al*. (2019) also assessed microplastics in water based on published data and found high variation between estimates, at least some of which could be attributed to variation in methods used to sample and quantify microplastics.

Bergmann *et al*. (2019), noting that microplastics are ubiquitous, assessed the likely importance of aerial transport in moving microplastics to areas where no known sources exist. They found that aerial transport played a significant role in microplastic transport and its availability in the air, soil, and water when the plastics were deposited. Aerial transport means that airborne microplastic may be inhaled by air breathers, including humans (Prata, 2018). Plastic microfibers are also accompanied in the air by other fibres, which could also be inhaled and may produce similar responses (Dris *et al*., 2017; Gasperi *et al*., 2018). Gasperi *et al*. (2018) cite research that reports around 30% of fibres are microplastic, with larger amounts found indoors. Most aerial microplastics found were microfibers (Kaya *et al*., 2018), with smaller particles predominating (Bergmann *et al*., 2019), suggesting that the number of particles and fibres smaller than analytic methods detection limits is significant. The majority of inhaled microfibers deposited on airways will be liable to mucociliary clearance, though some may remain, particularly where clearance mechanisms are compromised (Gasperi *et al*., 2018). If fibres are maintained, their biopersistence is liable to be high (Law *et al*., 1990). Fibre size also affects biopersistence, with larger fibres being more likely to be retained (Warheit *et al*., 2001). As a result, plastic fibres have been found in lung tissue (Pauly *et al*., 1998).

Microplastics have been found in marine bivalves cultured for human consumption (van Cauwenberghe and Janssen, 2014). Microplastics have also been found to accumulate in freshwater shellfish (Su *et al*., 2018). In the latter example, the Asiatic Clam *Corbicula fluminea* is consumed by humans without removal of the digestive tract, meaning that all microplastics accumulated by the clam are available to humans that consume it. Microplastics may be found in freshwater fish caught for human consumption (Biginagwa *et al*., 2016; Silva-Calvacanti *et al*., 2017; Ding *et al*., 2018).

The majority of microplastics reported from freshwater fish are recovered from the gut of freshwater fish, although microplastics can also be found in skin, gill and other tissue (Karbalei *et al*., 2018; Lei *et al*., 2018; Ding *et al*., 2018). When the digestive tract is removed before consumption, gut microplastics are also removed (EFSA, 2016). However, the fish meal prepared using non-food fish waste is used in poultry and pig feed and can enter other food sources that way (EFSA, 2016). It has been proposed that microplastic retention in fish guts is low (Jovanovíc, 2017), and therefore that, unlike shellfish, the microplastics consumed by fish are largely not transferred to human consumers. This research documents the number of microplastics that humans or other consumers of shellfish and fish might be exposed to due to microplastics in the fish or shellfish. However, Catarino *et al*. (2018) documented the

significance of microplastic deposition on fish during preparation and concluded that this is minimal than microplastic dust deposited on a meal during consumption. Microplastic levels also increase during food preparation (Renzi *et al*., 2018).

Microplastics are available to humans in several other ways. Microplastics have been found in salt (Iñiguez *et al*., 2017; Karami *et al*., 2017; Kosuth *et al*., 2018), honey (Mühlschlegel *et al*., 2017), beer (Kosuth *et al.,* 2018; Liebezeit and Leibezeit, 2014), tea (Hernandez *et al*., 2019), and sugar (Liebezeit and Leibezeit, 2013). Plastics might also be directly ingested through direct contact with plastic articles such as child's toys, plastic cups and plates, etc. (WHO, 2019). Cox *et al*. (2019) used data on microplastic presence in water, air, and other sources to estimate the number of microplastics that might be ingested by an American as being between 39000 to 52000 particles depending on age and sex. Inclusion of microplastics inhaled increased these estimates to 74000 and 121000. Intake of microplastics in water could increase exposure where bottled water is a significant part of water intake. The authors indicate that estimates reveal much variation but conclude that their estimates are likely to underestimate. Exposure was greater for adults in comparison with children. The majority of microplastic came from the air, followed by bottled water and seafood. Catarino *et al*. (2018) found the majority of microplastics on cooked mussels derived from falling household dust rather than the mussels themselves.

#### *2.2.3.2 Potential human health effects*

While intake of microplastics by humans through inhalation is not directly related to the freshwater focus of this project, inhalation of microplastics and the effects thereof will be included as the information presented here relates to impacts of microplastics when taken up by an exposed organism. Although awareness of airborne microplastics is relatively recent, and data on exposure are rare, variable and unreliable, occupational exposure to microplastic has seen more research (Prata, 2018). Occupational exposure studies deal more commonly with scenarios where exposure to larger amounts of microplastics for a shorter period of time might be expected from simple environmental exposure. Workers exposed to microplastics come predominantly from the synthetic textile, flocks, and polyvinylchloride industries (Prata, 2018). Diseases from microplastic inhalation range from asthma-like reactions, diffuse interstitial fibrosis and granulomas with fiber inclusions, inflammatory and fibrotic changes in the bronchial and peribronchial tissue, and interalveolar septa lesions (Prata, 2018). Additives within flock and polyvinyl chloride (PVC) can add to the scale of inflammation.

Workers in the textile industry have been found to have foreign-body-containing granulomatous lesions that were believed to contain microplastic dust (Pimental *et al*., 1975). Workers in this industry have reported a number of respiratory complaints (Warheit *et al*., 2001), although a link to cancer has not been established (Gasperi *et al*., 2018). Predictions of the impact of plastic microfibers based on experience with asbestos could potentially be made given that asbestos toxicity is modified by fibre length and biopersistence, despite the differences observed between respiratory problems in microfiber workers and workers exposed to asbestos (Gasperi *et al*., 2018).

The impacts of inhaled microplastics are largely inflammatory responses and cytotoxicity (Prata, 2018). The inflammatory response is associated with releasing intracellular messengers and cytotoxic factors, leading to the ongoing production of reactive oxygen species (ROS). Inflammatory lesions may result from several causes, which may develop into malignant lesions. ROS release can stimulate secondary genotoxicity (Gasperi *et al*., 2018). In this way, exposure to low concentrations of microplastics can cause gene mutation and cancer. Inhaled particles can also be translocated to other parts of the body and may have effects there (Prata, 2018).

Microplastics are primarily stable in human body tissue and are expected to be retained for long periods if not cleared (Gasperi *et al*., 2018). Longer fibres have a higher persistence than shorter ones (Gasperi *et al*., 2018). Retention of fibres allows for overall increases in fibre load with time. Longer fibres are also predicted to have a greater impact. As with all microplastics, inhaled microplastics may be associated with non-polymer chemicals that have the potential to leach from inhaled microplastics (Gasperi *et al*., 2018 and references therein). These can be pollutants adsorbed to the hydrophobic surface and plasticisers, colourants, and other compounds within the plastic. Negative impacts may also result from biota associated with the microplastics (Prata, 2018). These can have additional adverse effects when microplastics are inhaled and retained. This highlights the potential for impacts from microplastics to be a result of physical, chemical or biological stress.

Microplastics can enter the human body in several ways. Inhalation is discussed above, but microplastics can also enter the human body in food consumed or drinking water. Seafood, and shellfish, in particular, have been found to contain microplastics (Barboza *et al*., 2018c, Renzi *et al*., 2018; Lusher *et al*., 2017), as have bottled and tap water (Kosuth *et al*., 2018; Cox *et al*., 2019; Schymanski *et al*., 2018; Koelmans *et al*., 2019). In addition, airborne microplastics have been found to contaminate food during preparation (Catarino *et al*., 2018), and this highlights the likelihood that any food may contain microplastics. Research by Cox *et al*. (2019) indicates that exposure to microplastics in drinking water and seafood are similar for someone consuming a typical American diet.

Although the toxicity of microplastics that have been inhaled has been studied, relatively little is known about ingested microplastics (WHO, 2019; Wright and Kelly, 2017; Barboza *et al*., 2018c). WHO (2019) indicated that plastic polymers alone are relatively inert and pose a little hazard. However, the physical shape and adsorbed or leached chemicals may modify this assumption. WHO (2019) noted that no data assessing the impact of ingested microplastics could be found and that identified risks of ingestion are therefore inferential. Retention of microplastics in the gut is not clear, but is believed to be extremely limited (WHO, 2019; SAPEA, 2019), and Schwabl *et al*. (2019) showed the passage of a significant amount of microplastics through the gut. Most toxicity testing has focused on aquatic organisms, with limited studies on rats, mice and human cell lines (WHO, 2019). Several of the studies that have been undertaken looked at scenarios where exposure to microplastics had an impact, but their relevance, given the high microplastic doses administered in light of known environmental levels, was questionable (WHO, 2019; SAPEA, 2019; Wright and Kelly, 2017).

Some studies were not able to detect any impact of microplastics fed to rats (WHO, 2019). For example, Merski *et al*. (2008) found that rats fed ground polyethylene and polyethylene terephthalate fabric showed no adverse effects on up to 5% microplastics diet. Rafiee *et al*. (2018) found no statistically significant neurobehavioural change in rats fed with polystyrene nanoparticles, but highlight the possibility that statistically insignificant behavioural changes might impact a population. Particles can be taken up across the gut (Volkheimer, 1993; Eldridge *et al*., 1989; Carr *et al*., 2012), and the likelihood that microplastics could pass across the gut wall is therefore likely to be high. However, the mucus layer restricted the diffusion of latex microbeads (Bajka *et al*., 2015). Particles that are adsorbed can pass into blood vessels and lymph glands and then be transported throughout the body and even transplacentally into the fetal blood-stream (Volkheimer, 1993). As such, adsorbed microplastics are likely to have access to other tissue including the liver and the brain (Barboza *et al*., 2018c). Human cell lines exposed to microplastics show signs of oxidative stress (Schirinzi *et al*., 2017), indicating a possible threat to human health posed by absorbed microplastics. Little data on exposure rates and uptake rates (and polymer types and sizes thereof) exist, and estimates of the extent of the risk are still challenging to make (Wright and Kelly, 2017; Barboza *et al*., 2018c). Given that studies on microplastic exposure in mice indicate alteration of oxidative stress, energy and lipid metabolism, and also neurotoxic effects, reasons for concern are valid (Revel *et al*. 2018).

Another potential impact of microplastics in the human gut relates to the potential impacts of microplastics on the gut biome. The importance of the human microbiome for health is now well recognised (e.g. Schreiner *et al*., 2015; Blaser, 2014), and changes to the gut microbiome may have impacts on human health. Microplastics at various doses have been found to impact the gut microbiome in mice (Jin *et al*., 2019), springtails (Ju *et al*., 2019), fish (Wan *et al*., 2019; Qiao *et al*., 2019), oligochaetes (Zhu *et al*., 2018), and other taxa (Fackelmann and Sommer, 2019; Smith *et al*., 2018; Lu *et al*., 2019). Changes to the microbiome may be a way that chronic effects of microplastic exposure are caused (Fackelmann and Sommer, 2019), and are of concern to humans (Smith *et al*., 2018; Lu *et al*., 2019). WHO (2019) indicate that significant inter-species variation in impacts found between studies means the relevance of this particular impact in human needs an exploration.

Completely polymerised plastics alone are relatively inert. However, polymerisation is not complete during production, and residual monomers are left, leaching from the plastic. In addition, plastics also contain plasticisers, colourants, flame retardants, and other additives that are generally not covalently bound to the polymer (Tickner, 1999; Murphy, 2003; Hahladakis *et al*., 2018) may have toxic effects if leached. Microplastic particles are also hydrophobic and so can sorb other compounds onto their surface (GESAMP, 2015), and these, in turn, may present a hazard when microplastics are consumed. These include known toxins such as persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and organochlorine pesticides (WHO, 2019). Compounds with a lower molecular weight are more likely to leach faster from plastics (Hansen, 2013). Together with variation in size, shape, and polymer, these compounds complicate quantifying the hazard posed by microplastics, as consideration of the many potential additives and the exposure to the environment will modify the risk profile of a particular microplastic (WHO, 2019). The leaching of toxins from microplastics may lead to elevated levels in human consumers.

Biofilms on water pipes have been a potential threat to drinking water quality due to the irregular presence of pathogenic microorganisms within the biofilm (WHO, 2017). Likewise, microplastics generally are surrounded by a biofilm to form a plastisphere (Zettler, Mincer and Amaral-Zettler, 2013). Antibiotic resistance genes and metal resistance genes have been found in greater numbers and diversity in the plastisphere than the surrounding environment (Yang *et al*., 2019). Arias-Andres *et al*. (2018) found that microplastic colonisation increases plastid and gene exchange between bacteria. This has led to concerns that microplastic biofilms may contribute to the dispersal and consumption of pathogens and antibiotic-resistant bacteria. As an example, microplastics that pass through wastewater treatment works may be a means of dispersing pathogens present in wastewater treatment works (McCormick *et al*., 2016; Oberbeckmann *et al*., 2018). McCormick *et al*. (2016) found increased levels of *Pseudomonas* spp., *Burkholderiales incertae sedis*, and Campylobacteraceae on microplastics compared to other suspended materials in the water column. Transfer of pathogens in a biofilm with increased antibiotic resistance is an added concern.

### 2.2.4 Effects of plasticisers, monomers and other additives

A plasticiser is a compound that, when added to a polymer, makes the end product softer and more flexible (Cadogan and Howick, 2000; Wypynch, 2017). This is achieved through a decrease in glass-transition temperature of the polymer. There may be as many as 30000 compounds with the potential to act as plasticisers, but of these, only 100 plasticisers are produced, and only 50 of these are commercially important (Godwin, 2011). Around 90% of plasticisers are used in the production of polyvinyl chloride products, and most are phthalate ester plasticisers. Polyvinyl chloride naturally contains a limited level of native PVC crystallinity, which leads to a lack of flexibility and elasticity, and plasticisers are needed to render polyvinyl chloride suitable for use (Carroll *et al*., 2011). Plasticisers

are used in other polymers, too, including nylon, polyurethanes, polyolefins, acrylic polymers and others (Godwin, 2011).

Leaching of plasticisers from plastic polymers has commonly been noted in a process that introduces the plasticiser to the environment around the plastic and to embrittlement of the plastic (e.g. Kastner *et al*., 2012; Erythropel *et al*., 2014; Jacobson *et al*., 1977; Zhang and Chen, 2014). Plasticisers can leach when they have not formed chemical bonds with the polymer that they are dispersed in (Kastner *et al*., 2012). The introduction of the plasticiser to the environment may cause a chemical threat, and this would apply regardless of whether the threat is caused by the plasticiser itself or its breakdown products. For example, di-2-Ethylhexyl phthalate (DEHP) is phthalate plasticiser that is commonly added to polyvinyl chloride (PVC) to produce wire insulation, roofing, piping and other building material, as well as medical tubing, blood and intravenous bags, food packaging, clothing and children's toys (Horn *et al*., 2004; Shea, 2003). The use of these materials in housing, medical equipment, children's toys, etc. exposes people to plasticisers leaching from the polymer. Concern has been expressed more about the breakdown products of di-2-ethylhexyl phthalate, which may act as endocrine disruptors, than the polymer itself (Erythropel *et al*., 2014). Likewise, polyvinyl chloride in the environment can leach plasticiser which may have an effect on surrounding biota (Fromme *et al*., 2002).

Di-2-ethylhexyl phthalate is not the only plasticiser that has elicited alarm owing to potential effects on leaching. Another compound that has attracted attention in this way is Bisphenol A (BPA). Bisphenol A is used as a monomer in the production of polycarbonates and epoxy and is produced and used in large quantities (Vandenberg *et al*., 2007). Bisphenol A leaches from the compounds and can accumulate in man and in the environment (Fromme *et al*., 2002; Vandenberg *et al*., 2007). Bisphenol A is a xenoestrogen that can act as an endocrine disruptor and has been found in human studies to reach levels in human blood that reach or exceed levels found in cell culture to trigger molecular endpoints (Wetherill *et al*., 2007; Vandenberg *et al*., 2007). In the same way, it can be found in varying levels in the environment (Fromme *et al*., 2002), where it has biological activity (Staples *et al*., 1998).

In summary, plasticisers are compounds added to polymers to increase flexibility. If not chemically bound within the polymer, plasticisers and other additives can and do leach from the polymer to their immediate environment. Once they are in the environment, they may impact biota present, either directly or through their breakdown products. As a result, much of the potential for the chemical toxicity of microplastics is linked to the impacts of plasticisers. In the course of this research, we will assess the toxicology of three different and chemically unrelated plasticisers. These are calcium stearate, dibutyl phthalate, and Bisphenol-A. They are described below.

#### *2.2.4.1 Calcium stearate*

Calcium stearate is the calcium salt of stearic acid. It is a white waxy powder with limited solubility in water. Calcium stearate has several functions in polymers, including PVC costabilizer, release agent, and lubricating many polymers, among other roles (Wypych, 2017). Polymer strength and impact resistance is improved by the inclusion of calcium stearate, which indicates that calcium stearate acts as a plasticiser rather than a lubricant (Wypych, 2017). Calcium stearate is therefore found in many polymers, but particularly in polyvinyl chloride. However, beyond the polymer industry, calcium stearate has many other uses including as an ingredient in food and pharmaceutical products (Budavari, 1996; Ley, 2001), as well as a lubricant, use in waterproofing, and production of pulp and paper (Ley, 2001; NCBI, 2019a and refs therein).

The EPA classify calcium stearate as being used as adhesives and sealant chemicals, anti-adhesive agents, fillers, finishing agents, flame retardants, hydrophobic agent, intermediates, lubricants and

lubricant additives, neutralising agent, polymer stabiliser, processing aids, and surface active agents (NCBI, 2019a). As might be surmised by the inclusion of calcium stearate in cosmetics and foodstuffs, calcium stearate has not been found to be significantly toxic (NCBI, 2019a; NIOSH, 2019). In tests using mice and rats, the oral and peritoneal LC50 was found to be > 10 g/kg (NIOSH, 2019). If these data were applied to a 75 kg human, that would equate to 750 g, or 670 ml of calcium stearate. As stearic acid is naturally found in some foodstuffs, where it has been found to improve thrombogenic and atherogenic risk factor profiles in males (Kelly *et al*., 2001), a major toxic threat is unlikely. Calcium stearate is readily biodegradable in nature (ECHA, 2019). As a plasticiser, calcium stearate is potentially a compound that could leach from microplastics, and might, in sufficient quantity, pose a threat to aquatic biota.

#### *2.2.4.2 Dibutyl phthalate*

Dibutyl phthalate is a manufactured compound that is not naturally found in the environment. It is a yellow to clear oily liquid with no odour. It is only slightly soluble in water. It is a plasticiser used in polymer manufacture, and has several other roles in various manufacturing processes, notably as an ingredient in paints, hair spray, glue, carpet backings, insect repellents, nail polish and rocket fuel (ATSDR, 2019). It has also been found in cigarette smoke and is sometimes used in dental moulding materials, among other uses (NCBI, 2019b).

It is not classified as carcinogenic to humans despite many tests (212 tests recorded at NCBI, 2019b). It was also not found to be mutagenic to Salmonella typhimurium, although it induced mutations in mouse lymphoma cells (NCBI, 2019b)). Toxicity tests found toxic responses in a number of test taxa using a number of test endpoints (NCBI, 2019b). Reported toxicity specifically included aquatic organisms. Dibutyl phthalate levels are restricted in children's toys and certain child care articles because of toxicity concerns. Dibutyl phthalate is also a potential endocrine disruptor (CDC, 2009). Despite the reported toxicity, dibutyl phthalate is biodegradable, and has a biodegradation half-life in aerobic conditions of one to a few days, slightly longer if the system is anaerobic (NCBI, 2019b). However, observed toxic responses mean that there is the still the potential for toxic responses after leaching from polymers but before biodegradation.

#### *2.2.4.3 Bisphenol A*

Bisphenol A, or 4,4′-dihydroxy-2,2-diphenylpropane, is an organic compound widely used in the manufacture of plastics (Allard, 2014). It consists of two phenol rings connected by a single carbon carrying two methyl groups (Allard, 2014). It appears as a white to brown powder, or flakes, that are slightly water-soluble (NCBI, 2019c). Bisphenol A is used as a precursor during the manufacture of polycarbonates and epoxies and as an additive in polyvinyl chloride (Allard, 2014). 95% of Bisphenol produced is used in the production of polycarbonates and epoxies, and the rest is used for a number of other proposes, including phenoplast resins, unsaturated polyester resins, phenolic resins, polysulfone generation, modified polyamines, polyols, can linings, flame retardants, pipe linings, paper coatings, etc. (EU 2003). Items that contain or are coated with Bisphenol-A include water, milk, and babies bottles, piping and can liners, spectacles lenses, compact discs, and medical and dental equipment. Bisphenol A levels in wastewater draining from landfills were found to be high, and it could be detected in a range of other environments (Fromme *et al*., 2002).

Bisphenol A was found to have estrogenic activity in 1938 and later found to bind to mammalian estrogen receptors, though less powerfully than estradiol. More recently, to elicit an estrogenic response in cell cultures in nanomolar quantities (Allard, 2014 and references therein). Bisphenol A can also affect the metabolic, thyroid hormone, and androgen systems (Miyagawa *et al*., 2016). Bisphenol is an

antiandrogen, decreasing testosterone (Ye *et al*., 2014) while increasing estrogenic activity. Bisphenol A has been found in human tissue and environmental samples (Fromme *et al*., 2002; Vandenberg, 2007; Huang *et al*., 2012; Oehlmann *et al*., 2009). Bisphenol A was found to affect reproduction in all animal groups that were studied (Oehlmann *et al*., 2009). It also had negative impacts on development in crustaceans and amphibians and could be found to induce genetic aberrations (Oehlmann *et al*., 2009). Environmentally relevant levels of these compounds have led to detectable impacts on molluscs, crustaceans and amphibians, all of which are particularly sensitive (Oehlmann *et al*., 2008). Fish were found to be less sensitive. Bisphenol A has also exhibited toxicity in tests on various mammals, with an LD50 of between 2 and 6 $g.kg^{-1}$ (NCBI, 2019c). Bisphenol in the environment does undergo biodegradation, and in one assessment, 90% of the Bisphenol A in several treatments was removed in four days (Dorn *et al*., 1987).

## 2.3 METHODS FOR IDENTIFICATION AND QUANTIFICATION OF MICROPLASTICS

### 2.3.1 Overview

This section reviews works concerning the methods used for sampling, analysing and evaluating the ecotoxicity of microplastics in marine and freshwater environments. Most studies use extraction and separation techniques to analyse microplastics from surface water and sediments samples (Claessens *et al*., 2011; Masura *et al*., 2015; Hanvey *et al*., 2017). The review focused on microplastics particle size less than 5 mm since they fall within the classical definition of microplastics, and are thought to affect biota in the environment more. For example, smaller particle sizes have a higher potential to get stuck in living organisms' guts and penetrate tissues much more quickly than larger ones (Nature Nanotechnology, 2019). More than 50 studies on microplastics in surface water and sediments have been evaluated in Hanvey *et al*. (2017), Bouwman *et al*. (2018) and Dris *et al*. (2018), with data from different countries, including the USA, Japan, Singapore, Brazil, India and South Africa. Synthesis of these studies showed four main steps to measure microplastics in surface water and sediment samples: sampling, separation, extraction and quantification. Therefore, this review will look at each of these processes. Methods for ecotoxicological and ecological studies will also be reviewed to quantify the impacts of microplastics on freshwater surfaces and sediments.

### 2.3.2 Field sampling for microplastics

Potential sampling sites are selected to collect surface water and sediment samples to analyse microplastics. Accurate surface water and sediment collection sampling is the first critical step in quantifying microplastics. Appropriate site selection and sampling will significantly impact the quality of the sample, abundance, particle size, and type of polymer (Wright *et al*., 2013; Hanvey *et al*., 2017). Most studies reported that polypropylene (PP), polyethylene (PE) and polyvinyl chloride (PVC) are the major polymer types that are detected in water resources (Lambert *et al*., 2017; Rist and Hartmann, 2018). They found that polymer types can vary based on the source and location. Each study reviewed had applied a slightly different methodology for sediment sampling, and samples were collected from superficial or in-depth sediment (Carson *et al*., 2011; Woodall *et al*., 2014; Turra *et al*., 2014).

In Coppock *et al*. (2018), the superficial sediment sampling was conducted, the top 2cm sediment sampled was collected using multicores and used for microplastic extraction. A stainless steel measuring cup was used to sample coarse sand in different places. The samples were sealed in foil and transported to the laboratory. Many studies reported that sediment at depth has a greater percentage of microplastics than surface sediment (Turra *et al*., 2014; Woodall *et al*., 2014; Martin *et*

*al*., 2017). For example, in a study where sampling depth ranges from 5 cm to 25 cm, most plastics were in the deepest section (Carson *et al*., 2011). A study in the North Atlantic Ocean sampled 500 m and 3500 m, and the most abundant microplastic was found in the deepest section (Woodall *et al*., 2014).

For surface water sampling, wide areas of water are sampled using trawl nets with different mesh sizes, or large volumes of surface water (approximately 100 litres) are sampled by filtering using a known sieve size (Coyle *et al*., 2016; Eriksen *et al*., 2017; Bouwman *et al*., 2018). Different trawl nets (Manta, AVANI and DiSalvo) are used to sample surface water microplastics (Eriksen, 2017; Dris *et al*., 2018; Briggs *et al*., 2019). Manta trawl nets are nets with 335 µm mesh size and a rectangular aperture of 16 cm high and 61 cm wide. A Manta trawl net has two large wings that help floatation and surface retention of the front of the trawl (Eriksen *et al*., 2017; Briggs *et al*., 2019). The All-purpose Velocity Accelerated Net Instrument (AVANI) trawl net has a mesh size of 335 µm, with a rectangular aperture 60 cm high and 14 cm wide (Eriksen *et al*., 2017). AVANI has an aluminium plate that keeps the trawl at the sea surface while it is towed, and it can completely submerge under high tides or skim on the surface water (Eriksen *et al*., 2017). The DiSalvo neuston net has a 300 µm mesh size with an aperture of 40 cm high and 80 cm wide. The Disalvo neuston net has a PVC pipe that supports the net with floatation. The AVANI design is advantageous compared to other nets because it has a taller opening, and it can stand rougher seas than Manta and DiSalvo net (Eriksen, 2017). The AVANI is also more efficient because it can cover a large area due to high tow speeds.

In studies sampling surface water by filtering, different volumes of water were filtered. In Jiang *et al*. (2018) 30 L surface water samples were collected at 0-30 cm depth. The surface water sample was filtered using a 45 µm stainless sieve. All of the solids on the sieve were rinsed carefully into a 1 L glass jar with deionized water; a 5% formalin solution was used as a preservative. Three duplicate samples were collected at each sampling site. In Bouwman *et al*. (2018), the NOAA standard microplastic protocol was used: ninety litres of surface water was filtered through a 20 µm sieve. Rinsed water was stored in pre-cleaned HDPE bottles and transported to the laboratory.

### 2.3.3 Extraction of microplastics from environmental media

The most commonly used method for extracting microplastics from sediments is density separation, which was developed by Thompson *et al*. (2004). Density separation uses water with brine solutions, for example, sodium chloride (NaCl), sodium bromide (NaBr), sodium iodide (NaI) and zinc bromide ($ZnBr_2$). Polypropylene and polyethylene are less dense than water, so they float in natural water, while polyvinyl chloride is denser than water and floats only when the density of water is increased. A mass of the selected salts is dissolved in 1 L of distilled water and stirred using a magnetic stirrer until no more salt is present at the bottom of the beaker. The following formula defines the density of the solution:

$$Density = \frac{Salt\ mass}{Water\ volume}$$

Different densities of the solutions are listed in Table 2-2. The solution density was checked often using polyethylene as a reference plastic (Thompson *et al*., 2004). A coffee grinder and a food processor were used to break down the secondary microplastics. A sieve was used to divide the microplastics into classes with different size ranges of 200-400 µm and 800-1000 µm. Fourier transform infrared spectroscopy (FTIR) analysis confirmed the sample identification. In Masura *et al*. (2015), a density separation was used, using sodium chloride to isolate the plastic debris through flotation. Bouwman *et*

*al*. (2018) used density separation in sample processing, but in the place of sodium chloride, iodine chloride was used (Bouwman *et al*., 2018).

**Table 2.2: Different of salts to prepare floatation solution for microplastics. Data from Thompson *et al.* (2004) and Coppock *et al.* (2017)**

| Full name | Chemical name | Density ($g \cdot L^{-1}$) |
|---|---|---|
| Sodium bromide | NaBr | 1.5 |
| Sodium chloride | NaCl | 1.2 |
| Sodium iodide | NaI | 1.3-1.5 |
| Zinc bromide | $ZnBr_2$ | 1.3-1.8 |
| Zinc chloride | $ZnCl_2$ | 1.3-1.8 |

On-site sieving is also widely used, even though it is only suitable for microplastics greater than 1 mm, while density separation is used for particle sizes less than 1 mm (Masura *et al*., 2015; Bouwman *et al*., 2018; Briggs *et al*., 2019). With on-site sieving, a known sieve size is used to sieve the sediment and for plastic particles.

Density separation of microplastics from sediment follows these common steps:

1. Sediment is dried in an oven. Different times and temperatures have been used for this purpose. For example, Coppock *et al*. (2017) dried the sediment at 50°C for 72 hours, Bouwman *et al*. (2018) dried the sediment at 90°C until dried, and Masura *et al*. (2015) dried the sediment at 90°C for 24 hours. The general trend is that drying sediment at higher temperatures requires less time and vice versa.
2. The dried sediment is mixed with salt solution then shaken or centrifuged to detach the polymer from the matrix.
3. The mixture is left to settle. Although the time allowed for settling was not generally reported, Coppock *et al*. (2017) left the mixture for 5 minutes to settle.
4. The solution is then filtered using Whatman filter paper 11 µm or GF/C filters with a pore size of 10 µm (Jiang *et al*., 2018; Mai *et al*., 2018; Blair *et al*., 2019).
5. The smaller microplastics need further observation under a microscope to identify the shape and colour and measure the size.

### 2.3.4 Separation

A commonly used technique for separating microplastics from surface water samples is vacuum filtration. To separate polymer from the matrix is an arduous process, and various oxidation techniques are used to eliminate organic matter (Debellefontaine *et al*., 1996). Liquid phase oxidation solutions such as hydrogen peroxide and wet peroxide oxidation are used to separate polymer from their matrix by digesting organic matter. Thirty percent (30%) hydrogen peroxide has been found to be the best solution to digest samples with higher organic matter since it does not alter the polymer's original shape and size. Although the process of digestion is critical, some of the studies do not report on it (Coppock *et al*., 2017; Woodall *et al*., 2018). Consideration should be considered since inaccurate digestion can lead to incorrect results.

### 2.3.5 Identification

Microscopy and visual sorting of microplastics are commonly used techniques for identification, though it has limitations. For example, visual counting can include non-polymer particles and give inaccurate particle numbers and size ranges estimates. Several studies reported that some of the particles identified as microplastics during the visual sorting later were identified as other compounds (Thompson *et al*., 2004; Hanvey *et al*., 2017; Bouwman *et al*., 2018). Different techniques, including Raman Spectroscopy, Fourier Transform-Infrared Spectroscopy (FT-IR), Scanning Electron Microscopy (SEM), Gas Chromatography/Mass Spectrometry (GC/MS), are used to confirm the presence of microplastics and their characteristics. It is advantageous to use more than one of the above techniques for the characterisation of microplastics. Raman Spectroscopy can provide structural information of the plastics that make it easy to determine the polymer type (Bouwman *et al*., 2018; Rist and Hartmann, 2018; Briggs *et al*., 2019). Fourier Transform-Infrared Spectroscopy is the most commonly used technique to identify microplastics for both surface water and sediments.

Characterisation by FT-IR facilitates colour identification, chemical composition, size, shape, morphology and structure of the polymer (Dris *et al*., 2018). Its non-invasive nature ensures that it does not change or destroy the sample during analysis. Scanning Electron Microscopy can identify inorganic plastics additives and give information about the shape and size of the particles (Hanvey *et al*., 2017). Using the combination of SEM and a technique such as FT-IR is advantageous as it gives information about the source of the plastic particles (Cooper and Corcoran, 2010; Fries *et al*., 2013; Zbyszewski *et al*., 2014). Gas Chromatography (GC) and Mass Spectroscopy (MS) are powerful techniques for identifying polymer typology by obtaining structural information about macromolecules (Hanvey *et al*., 2017). The limitation of these techniques is that they do not give any information on morphology, type, and the number of plastics present in samples (Corcoran *et al*., 2009).

## 2.4 EXPERIMENTAL DESIGN CONSIDERATIONS FOR ASSESSING THE AQUATIC ECOTOXICOLOGY OF MICROPLASTICS

### 2.4.1 Controls

Different types of controls are used in ecotoxicological experiments. For a negative control, the test organisms are not exposed to the test substance, whereas a positive control involves exposing a group of organisms to a substance, either than the test substance, known to produce an effect. Solvent or vehicle control is used when the test substance is dissolved in a solvent or a vehicle before it can be administered successfully. In such experiments, two control groups are included of which one receives only what is in the natural laboratory environment (e.g. dilution water in an aquatic experiment, a water spray in a pesticide application experiment, and unadulterated food in a feeding study), while the other group receives the dilution water with added solvent but no test substance. Microplastics could either be administered directly through the water or sediment medium or food could be used as a vehicle in exposure experiments.

### 2.4.2 Treatments

Generally, ecotoxicological experiments involve one or more treatment groups and a control. Treatment groups differ only in the amount of the test substance to which the subjects are exposed, but all other conditions are nearly kept the same. Thus, aside the amount of test substance, factors such as test species, strain, age, sex, ambient conditions, and diets should be the same in all treatment groups and

control. In this instance, treatment groups might be tanks of fish exposed to different concentrations of the microplastics.

### 2.4.3 Replication

Replication is inevitable in ecotoxicological experiments because of the inherent variability in measurements on living organisms. A replicate (also an experimental unit) is the basic unit of organisation of test subjects that have the same ambient conditions and exposure to the test substance. (Green *et al*., 2018). Different replicates receive different treatments by randomisation to ensure that each replicate captures all the sources of variability in the experiment other than the level of substance exposure. Each treatment group and control are replicated to ensure that multiple subjects are exposed to each group. Since two animals exposed to the same test substance would not necessarily have the same sensitivity to that substance, replication separates the inherent variability among subjects (if any) from the effects of the test substance. The number of replicates and the number of subjects per replicate influence the power in hypothesis testing and the confidence limits of parameter estimates and other model evaluation measures in regression models.

The number of subgroups per concentration and subjects per subgroup should be chosen to provide adequate power to detect an effect of magnitude deemed necessary to detect or to result in a slope or ECx estimate with acceptably tight confidence bounds. Generally, increasing the number of replicates and reducing the number of subjects per replicate will lead to greater power or sensitivity due to a slope or ECx estimate with narrow confidence intervals. Generally, (1) if the variance between subjects greatly exceeds the variance between replicates, then greater power or sensitivity is usually gained by increasing the number of subjects per replicate, and reducing the number of replicates but never less than two per treatment. Otherwise, greater power or sensitivity generally comes from increasing the number of replicates and reducing the number of subjects per replicate. (2) There need to be more replicates per treatment and fewer treatments for hypothesis testing, whereas it is better to have more treatments and less replicates for regression analysis. For microplastics experiment, a minimum of two subgroups per concentration is recommended, although three subgroups are much better than two, and four subgroups better than three.

### 2.4.4 Choice and spacing of test concentrations

The number and spacing of concentrations are important factors worth considering when developing exposure experimental designs so as to provide adequate power to detect effects that are of a magnitude deemed biologically important. The goal of choosing test substance concentrations is to bracket the concentration at which biologically important effects appear, while spacing the levels of the test concentrations as closely as practical. If limited toxicity information exists for a particular microplastic, the exposure levels can be selected to cover a range somewhat greater than levels expected to be encountered in the field and should include at least one concentration expected not to have a biologically important effect. However, the range may be reduced if adequate information is available so that concentrations can be spaced more closely. Judgement on the exposure levels expected to produce an effect of interest can be made through literature review, or a small range-finding may be conducted before a larger definitive one. Effects are usually expected to increase approximately in proportion to the log of concentration, so concentrations are generally approximately equally spaced on a log scale. It is suggested that three to seven concentrations and appropriate controls are used.

### 2.4.5 Model microplastic compounds

Polypropylene (PP), polyethylene (PE) and polyvinyl chloride PVC beads and fibres are commonly used for toxicity testing. Recent research used these microplastics because they were identified as the most frequently used and also abundant in water resources (Au *et al*., 2015; Lambert *et al*., 2017). Particle shape was found to significantly affect the organism (Au *et al*., 2015; Lambert *et al*., 2017). For example, PP fibres have higher toxicity than PP beads (Au *et al*., 2015; Lambert *et al*., 2017; Nature Nanotechnology, 2019). Unevenly shaped, granular, needle-like, or fibre microplastics exact more effect on organisms. It is noteworthy that the crystallinity of microplastics consists of tightly structured and more ordered polymer chains. These characteristics affect the permeability and density of microplastics. Further, crystallinity changes with degradation time, leading to differences between environmental microplastics and their microbeads. These changes could influence particle size, shape, density and surface area (Lambert *et al*., 2017).

### 2.4.6 Test organisms

Microplastics are mainly found in the water column and sediments, rendering organisms that inhabit these spaces vulnerable to toxicity effects from such microplastics. In South Africa, candidate test organisms used in laboratory experiments include zebrafish and tilapia, freshwater snails and freshwater shrimps.

### 2.4.7 Statistical considerations

Two commonly statistical tests used in ecotoxicology that are applicable to microplastic exposure experiments. These are t-test, which is commonly applied for comparison of only two samples from a statistical population, and multiple hypothesis tests, such as analysis of variance (ANOVA), followed by multiple comparison tests, commonly used for comparisons between multiple samples (for example a control group and more than one treatment groups for a given time point). Traditionally, the no observed effect concentration (NOEC) has been determined using hypothesis testing, but this practice has been criticised because of perceived deficiencies (Fox and Landis, 2016). These deficiencies include the fact that (a) it is one of the test concentrations (b) the procedure by which it is determined “rewards bad experiments” (c) it cannot be determined in some cases (d) its size is a function of the choice of statistical test and level of significance, and therefore (e) definite conclusions cannot be made based on it. Thus, over the last two decades, there has been calls to replace the use of hypothesis testing to determine NOECs by regression models to estimate specific percent effects concentration, ECx. One goal of the regression approach is to replace the ill-defined connection between biological and statistical significance with an estimate of the exposure level that produces an effect of a specific size.

Every statistical test used to derive a NOEC or estimate an ECx has an underlying model. A fundamental experimental design in ecotoxicity is one in which independent groups of subjects are exposed to different concentrations of a single test substance for the same length of time, so that the only non-random source of difference among test organisms is the levels of exposure to the test substance. For most species, it is expected that an existence of an effect of the test substance will tend to increase as the test substance concentration increases. This fundamental experimental design may be represented by the statistical model

$$Yij = \mu i + eij \qquad (1)$$

where μi is the expected mean response in the ith concentration, and eij are independent identically distributed random errors, often assumed to be normally distributed with homogeneous variances, though that is not by any means an absolute requirement. Additional restrictions or assumptions may be placed on the treatment means (μi) in order to distinguish one model from another.

For hypothesis testing, the model is usually stated in terms of null and alternative hypotheses in the form

$$H03: \mu 0 \geq \mu 1 \geq \mu 2 \geq \cdots \geq \mu k \text{ vs. } Ha3: \mu 0 > \mu i \text{ for some I,} \quad (2)$$

where μ0 is the control mean. This model assumes a non-increasing concentration-response, which is what is expected biologically for most responses from ecotoxicity experiments.

Regression models assume a specific mathematical form for the relationship between treatment mean and concentration. For example, when modelling length or weight of fish from an aquatic experiment, one might hypothesise

$$\mu i = aebxi \quad (3)$$

where xi is the concentration in the ith treatment, and a and b are positive parameters to be estimated from the data.

## 2.5 SUMMARY

The research presented above is a limited data set, as studies are often focused on particular groups. For example, in feeding studies, most data are from fish, bivalves and crustaceans, and all the behavioural data found for this review referred to fish. This provides a limited dataset to infer ecological impacts from the studies undertaken. Very few algal studies could be located, despite making up a significant part of the ecosystem. Data on microplastic impacts on protists is likewise scarce.

Nearly all the studies cited here looked at single-species responses. As such, it may be possible to infer the general impact of microplastics and nanoplastic from the data in studies published to date, no data on community-level impacts were found to support any inferences made. Many of the studies were relatively short-term, and little information is available about the longer-term impacts of these contaminants. The nature of the pollutants is likewise not explicit, as different polymers may have other impacts, different sizes and shapes of polymers may have different impacts, and the interaction between particles and adsorbed contaminants are complex, and responses to particles with biofilm (as would occur in the wild) may change too. Nevertheless, it is important to bear in mind that although a great number of microplastic and nanoplastic impacts have been identified, several studies that were undertaken did not report impacts. It is also important to note that many of the studies that were reviewed above were undertaken at unrealistically high concentrations of particles, and the results reported may be unrealistic in the natural environment where particle levels may be lower (SAPEA, 2019; Triebskorn *et al*., 2019; GESAMP, 2016; Tang, 2017; Lenz *et al*., 2016).

# CHAPTER 3: ASSESSING THE TOXICITY OF MICROPLASTICS AS A PHYSICAL STRESSOR ON SELECTED FRESHWATER ORGANISMS

## 3.1 INTRODUCTION

Emerging contaminants, including plastics, is among the leading pollutants impacting surface waters globally, causing biodiversity decline, ecosystem impairment and human health risks (Ogo *et al*., 2022; Andrady 2011). Plastic pollution is largely triggered by global industrialisation and urbanization, leading to extreme global plastics production and other emerging environmental contaminants (Wang *et al*., 2021). Plastic pollution is expected to increase at an alarming rate from the projected increase in plastic production to compensate for the plastic needs of the projected population growth globally (Lourenço *et al*., 2017; Naidoo, Rajkaran, and Sershen 2020). The proliferation of plastics in water bodies has become a serious environmental concern, with significant ecological impacts through entanglement and ingestion, with indirect impacts owing to habitat damage (Abduro Ogo *et al*., 2022; Miao *et al*., 2021). Plastic materials gradually disintegrate by combining biological and environmental factors into tiny microplastics particles, posing physical stress on aquatic organisms (Carson *et al*., 2013; Eriksen *et al*., 2021).

Microplastics are plastic particles from <5 mm and can be classified into primary and secondary (Merga *et al*., 2020; Wu *et al*., 2021). Primary microplastics include microbeads, industrial cleaners, and personal care products such as toothpaste, facial and body scrubs, laundry detergents, sunscreens and drug containers (Liu *et al*., 2021). At the same time, secondary microplastics are formed by the degradation of large to microscopic particles by a combination of biological and environmental factors such as mechanical abrasion by sand or water, photodegradation, biodegradation and temperature (Ogo *et al*., 2022). Generally, microplastic is characterized by having a large surface area to volume ratio, chemically diverse and easily transported long-distances, persist in the environment, and easily ingested by aquatic organisms, and then enter higher trophic organisms through the food chain (Bulannga and Schmidt 2022; Kumar *et al*., 2021). Thus, the synergistic effect of microplastics and other contaminants can lead to serious impacts (e.g. death and risks to organs) on fishes (e.g. zebrafish) (Chen, Li, and Li 2021). The investigation on the toxicity of microplastics in the aquatic environment has become important in recent years. However, the effects of microplastics on biochemical traits of organisms remain poorly understood.

Risks posed by exposure to microplastics may result from several microplastic characteristics, including the physical properties of microplastics particles, chemical toxicity of plastic components, chemical toxicity of compounds adsorbed to the microplastic particles, and interactions between any of these factors. As a result, microplastics can severely impact aquatic organisms by causing physical damages to organisms and toxicity effects (Liu *et al*., 2021; Zhang *et al*., 2022; Zhao *et al*., 2021). In freshwater environments such as rivers and streams, organisms are constantly exposed to a range of plastic types and particle sizes and shapes (Windsor *et al*., 2018; Wu *et al*., 2021), increasing their venerability to stress from plastics particle. Plastics can affect organisms across various organisational levels, from the cellular, tissue, and organ levels to the complete organism. Microplastic effects ranging from molecular and cellular, feeding behaviour through ingestion, and inflammation of tissues and organs through adsorption have been documented (Windsor *et al*., 2018).

It is necessary to conduct an in-depth investigation on evaluating and reducing the risks of microplastics to aquatic organisms. This topic has been barely tackled before. Therefore, this chapter explores the toxicity of microplastic polymers on selected freshwater organisms under different exposure conditions.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Taxa used for assessing microplastics toxicity

In this study, taxa used in toxicity tests included the snail *Melanoides tuberculata*, shrimp *Caridina nilotica* as well as fishes *Tilapia sparrmanii* and *Danio rerio*. These organisms were collected from different sources, including the Bushman River, the Rivendell hatchery, and laboratory cultures maintained in Unilever Centre for Environmental Water Quality (UCEWQ) laboratories (Table 3-1).

**Table 3.1: Characteristics of test taxa utilised in laboratory toxicity tests**

| Scientific name | Common name | Family | Age | Source |
|---|---|---|---|---|
| *Melanoides tuberculata* | Snail | Thiaridae | Adult | Cultured at IWR labs |
| *Caridina nilotica* | Shrimp | Atyidae | Juvenile | Cultured at IWR labs |
| *Tilapia sparrmanii* | Tilapia | Cichlidae | Juvenile | Rivendell hatchery |
| *Danio rerio* | Zebrafish | Cyprinidae | Juvenile | Cultured at IWR labs |

### 3.2.2 Microplastic preparation

Ground microplastics were sieved using a 2.5 mm mesh to remove larger particles, and then test suspensions were prepared using 0.5 ml of ground, sieved microplastics in a fixed volume of water. Samples of microplastics were examined under a microscope to assess microplastic shapes and size distribution (Table 3-2). Microplastics' test suspensions were prepared using a serial dilution of microplastic suspensions with dechlorinated tap water (Table 3-3). Control exposures used during tests also used dechlorinated water. The range of microplastic concentrations tested accords relatively well with reported environmental levels, as Li *et al*. (2020) report 320 particles/L for South Africa, with maximum global levels reported at approximately 3000 particles/L. However, Bouwman *et al*. (2018) reported lower levels from South Africa. Only polypropylene, which produced smaller particles than the other microplastics tested, had unusually high levels of microplastics at the top of its range.

**Table 3.2: Size and shape of microplastic particles used in toxicological tests.**

| Shapes | Polypropylene | Polyethylene | Polyvinyl chloride |
|---|---|---|---|
| Fibres (%) | 30 | 65 | 20 |
| Spheres (%) | 20 | 15 | 30 |
| Irregular (%) | 50 | 20 | 50 |
| **Size** | | | |
| Avg length (µm) | 18.4 | 29.3 | 24.8 |
| Range (µm) | 1.7-1400 | 10-2500 | 2.9-1600 |

**Table 3.3: Concentrations of microplastic particles used in toxicological tests.**

| Microplastic | Concentration gradient (particles/L) | | | |
|---|---|---|---|---|
| Polypropylene | 0 | 1566 | 3131 | 6263 |
| Polyethylene | 0 | 923 | 1846 | 3691 |
| Polyvinyl chloride | 0 | 720 | 1440 | 2880 |

### 3.2.3 Toxicity testing

Static renewal experimental methods were employed for all chronic tests reported on here. Test organisms were maintained in solutions containing varying levels of the toxicant in question, and the solution was replaced at regular five-day intervals. All exposure tests used the same water as controls. All animals were exposed to a concentration gradient of the selected toxicant. For all animal exposure tests, each concentration was replicated three times. Tilapia and zebrafish were fed with TetraMin tropical flakes to satiation twice a day during the experimental period. Snails were fed with Spirulina powder two times a day during the long-term experiments. Experiments were conducted under a 12-hour light: 12-hour dark artificial light regime using Biolux fluorescent tubes in a temperature-controlled room at 25ºC (± 0.05ºC) in 600-mL glass beakers. The experimental chambers were provided with aeration, and the temperature was monitored. Water quality parameters such as pH, dissolved oxygen (DO), electrical conductivity (EC) and temperature were measured in all concentrations at the beginning of the experiment and every five days. Observations such as survival, reproduction, movement, ingestion and shredding were recorded every day. Table 3-4 shows the endpoints selected for toxicological tests of reproduction and growth.

**Table 3.4: Endpoints selected for toxicological tests of reproduction and growth**

| Test | Endpoint |
|---|---|
| Snail reproduction test (15 days) | Offspring produced per adult (two or five per replicate) |
| Snail reproduction test (21 days) | Offspring produced per adult (two or five per replicate) |
| Snail growth test-adult (15 days) | Individual adult shell length in mm. |
| Snail growth test-adult (21 days) | Individual adult shell length in mm. |
| Snail growth test-offspring (15 days) | Individual offspring shell length in mm. |
| Snail growth test-offspring (21 days) | Individual offspring shell length in mm. |
| Shrimp growth (21 days) | Length of juvenile in mm |
| Tilapia growth (21 days) | Length of juvenile in mm |

### 3.2.4 Data Analyses

Endpoints where data were normally distributed, were compared using linear model analysis. A Chi-squared test compared the generated model with a null model to evaluate model significance. The significance of individual parameters was determined using a Type II analysis of deviance, and pairwise comparisons used a Tukey adjusted pairwise comparison of estimated marginal means. The $EC_{50}$, or concentration where 50% of the test population were affected at the end of the test, was assessed where possible after fitting log-logistic models to data. Three-parameter log-logistic fits were most commonly applied. In some cases, the data suggested that other models might be more appropriate, and these were applied as necessary. Where data had a continuous distribution, a standard lack-of-fit test was applied to confirm the fit of the generated dose-response curve to the data (Bates and Watts,

1988). All statistics and plotting used R 4.0.3 (R core team 2020), together with the libraries drc (Ritz *et al*., 2015), car (Fox and Weisberg, 2019), lmtest (Zeileis and Hothorn, 2002), emmeans (Lenth, 2020) and plyr (Wickham, 2011).

## 3.3 RESULTS

### 3.3.1 Sensitivity of the selected aquatic organisms to different types of microplastics

#### *3.3.1.1 Snail reproduction tests*

The snail reproduction test could not distinguish between different microplastics (p=0.783), and was even less sensitive to differing concentrations of microplastics tested (p=0.855). Overall, the assigned model was not significantly different to a null model (p=0.901), and as a result, no impacts of microplastics on snail reproduction were detected. The snail growth test for adults was also unable to distinguish between microplastics or the concentrations of microplastics (p=0.508 and p=0.114, respectively). Given that no significant changes due to microplastics were found, it is not surprising that the test model cannot be distinguished from a null model (p=0.199). Therefore, there is no overall impact of microplastic particles on adult snail growth could be found. The impact of differing microplastics on the growth of snail offspring was assessed as it is possible that younger snails might be affected more. These results were similar to the same test using adult snails, and neither microplastic type nor concentrations of microplastics resulted in statistically significant changes in young snail growth (p=0.523 and p=0.402, respectively). As with the test using adults, it is unsurprising that the test model cannot be distinguished from a null model (p=0.487). Therefore, no significant impact of microplastics on young snail growth was detected.

#### *3.3.1.2 Tilapia growth tests*

As in the tests on snails reported above, neither microplastic type (p=0.368) nor the concentration of microplastics (p=0.788) could be statistically linked to variation in juvenile *Tilapia* body length after 21 days' exposure. As a result, the overall statistical model could not be clearly distinguished from a null model (p=0.640). Consequently, no significant changes to juvenile *Tilapia* growth because of microplastic exposure were found. Given that microplastic exposure had no significant impact on growth measured by a body length in juvenile *Tilapia*, it is of little surprise that the results measured by body width led to the same conclusions. Exposure to microplastics for 21 days did not lead to changes in juvenile *Tilapia* body width due to microplastic type (p=0.440) or concentration (p=0.750). The overall statistical model generated was not significantly different to a null model (0.682); as a result, the impacts of microplastics were not detected. The final test that assessed growth in juvenile *Tilapia* exposed to microplastics for 21 days used mass to measure growth. Unsurprisingly, given the results from tests using other growth measures above, growth by mass of juvenile *Tilapia* exposed to microplastics for 21 days showed no clear response to either microplastic type (p=0.972) or concentration (p=0.223). The statistical model was not distinguishable from a null model (p=0.574), and as a result, no impact of microplastics on *Tilapia* growth were found.

### 3.3.2 Assessing the toxicity effects of different microplastic concentrations-Dose-response relations

Data from each toxicity test was used to fit dose-response curves to characterise the test taxa's response to the microplastics assessed. Dose-response curves mainly were log-logistic, though relations such as shifted asymptotic sometimes fitted the data better. The $EC_{50}$ and $EC_{10}$ estimates from the fitted curves are presented in Table 3-5 and Table 3-6. These estimates are the concentrations of microplastics that will impact 50% or 10% of the test taxon. Inspection of the data in Table 3.5 and Table 3-6 reveal the substantial standard errors around nearly all the $EC_{50}$ and $EC_{10}$ estimates. This is a result of the data showing no clear toxicological response to any of the microplastics in the tests that were undertaken. In all but one case, the standard errors were greater than the estimated values, implying that the $EC_{50}$ and $EC_{10}$ values presented in the tables are not statistically supported and therefore, using the microplastics at the concentrations tested, no significant toxicological response was found (with the possible exception of the effects of polypropylene particles on fish growth). The statistical results will be addressed in greater detail below when the results of the individual tests are presented.

**Table 3.5: $EC_{50}$ data from toxicity tests using the microplastics polypropylene, polyethylene and polyvinyl chloride. $EC_{50}$ data are presented with standard errors. NA indicates that the curve-fitting algorithm failed to fit a curve to the data.**

| | Microplastic | | |
|---|---|---|---|
| **Test** | **PP (particles/L)** | **PE (particles/L)** | **PVC (particles/L)** |
| Snail reproduction test (21 days) | NA | NA | 3306±53809 |
| Snail growth test-adult (21 days) | NA | 15963±214039 | 66870±343122 |
| Snail growth test-offspring (21 days) | 34539±290992 | 2418±3927 | 16404±133622 |
| Tilapia growth juvenile length (21 days) | 518±1252 | 53895±696739 | 318600±9221441 |
| Tilapia growth juvenile width (21 days) | 22±7 | 8238±35805 | 27448±1230800 |
| Tilapia growth juvenile mass (21 days) | 177632±4809900 | 19622±103247 | 27370±120311 |
| Particles egested by juvenile Tilapia (21 days) | 656±13502 | 1321±4663 | 1038±5404 |

**Table 3.6: $EC_{10}$ data from toxicity tests using the microplastics polypropylene, polyethylene and polyvinyl chloride. $EC_{10}$ data are presented with standard errors. NA indicates that the curve-fitting algorithm failed to fit a curve to the data.**

| | Microplastic | | |
|---|---|---|---|
| **Test** | **PP (particles/L)** | **PE (particles/L)** | **PVC (particles/L)** |
| Snail reproduction test (21 days) | NA | NA | 2468±37440 |
| Snail growth test-adult (21 days) | NA | 12034±134016 | 7430±38125 |
| Snail growth test-offspring (21 days) | 5250±44232 | 1193±3440 | 1823±14847 |
| Tilapia growth juvenile length (21 days) | 79±190 | 8192±105907 | 48428±1401688 |
| Tilapia growth juvenile width (21 days) | 3±1 | 915±3978 | NA |
| Tilapia growth juvenile mass (21 days) | 85800±1894576 | 2983±15694 | 3041±13368 |
| Particles egested by juvenile Tilapia (21 days) | 464±7328 | 201±709 | 158±822 |

### 3.3.3 Effect of different microplastic types and concentrations on snails

#### *3.3.3.1 Snail reproduction test*

Figures 3-1 to 3-3 show successful reproduction in a freshwater snail in polypropylene, polyethylene and polyvinyl chloride suspensions of varying strength. For polypropylene (Figure 3-1), the model-fitting process could not resolve two out of three parameters, and no clear response to the microplastic at the concentrations assessed is seen. Figure 3-2 shows successful snail reproduction in varying concentrations of a polyethylene suspension. The model-fitting algorithm could not converge on any model so that no dose-response curve could be plotted. Inspection of the data in the figure does not indicate any clear response to the microplastic.

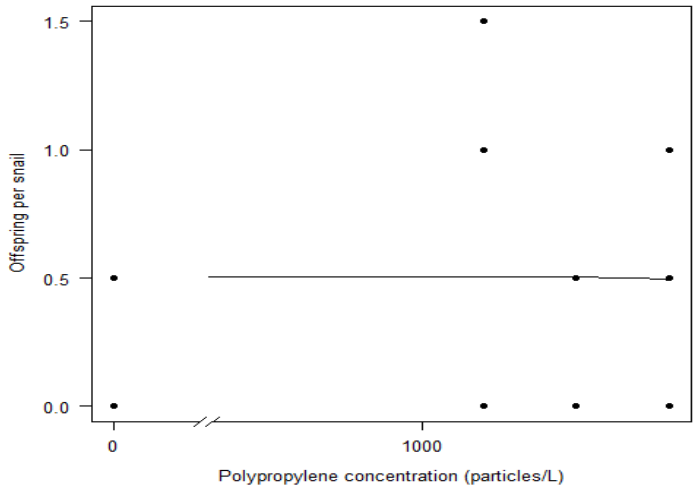



**Figure 3-1: Dose-response curve showing offspring production per adult snail in varying concentrations of polypropylene suspensions.**

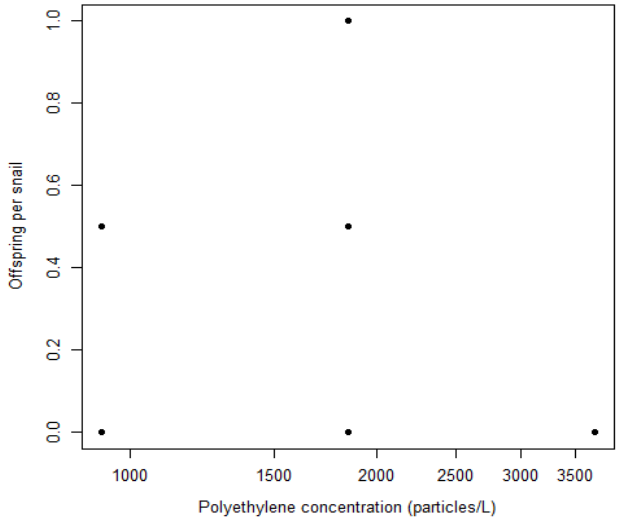



**Figure 3-2: Dose-response curve showing offspring production per adult snail in varying concentrations of polyethylene suspensions.**

Figure 3-3 shows the number of offspring produced by each adult snail exposed to varying concentrations of a polyvinyl chloride suspension. The model-fitting algorithm could only estimate four curve parameters at a statistically significant level. Assessment of the fitted curve shows no clear response to the microplastic across the concentration range tested. However, there is a suggestion that response might occur at higher concentrations.

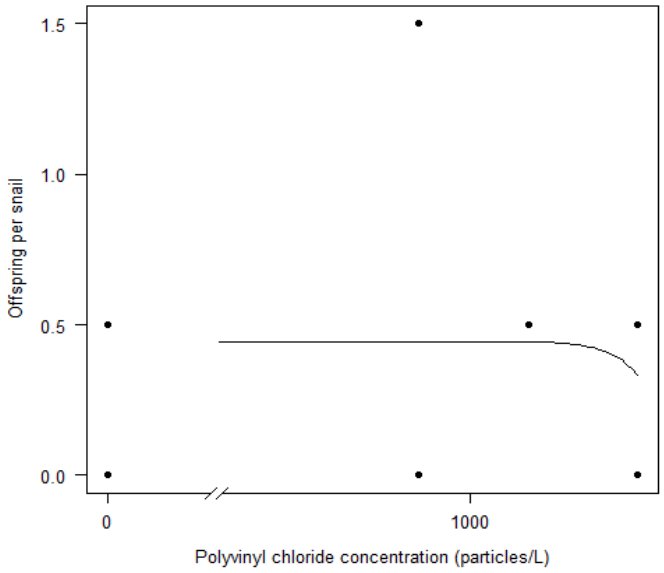



**Figure 3-3: Dose-response curve showing offspring production per adult snail in varying concentrations of polyvinyl chloride suspensions.**

### *3.3.3.2 Adult snail growth test*

In Figure 3-4, the response of snail growth, measured as shell width in adult snails after 21 days of exposure to varying concentrations of a polypropylene suspension. The fitted curve shows no response to the range of concentrations of polypropylene tested. The curve fitting procedure fitted all curve parameters significantly, and the curve seems to describe the data (p=0.092) adequately. The results from assessments of snail growth in polyethylene show similar results to those from polypropylene (Figure 3-5). The data presented in the figure show no response to the levels of polyethylene tested. Curve fitting procedures could only fit one of three curve parameters. The data and fitted curve relating snail growth after 21 days to varying levels of polyvinyl chlorine are presented in Figure 3-6. The curve in Figure 3-6 suggests that growth in *M. tuberculata* decreased as polyvinyl chloride levels increased. Still, an inspection of the raw data indicates that this is the effect of a few outliers and is not supported by the results in the range of polyvinyl chloride suspensions tested. The curve-fitting process could only reliably estimate one of three curve parameters, and a lack-of-fitness test did not find a good fit to the data (p=0.293).

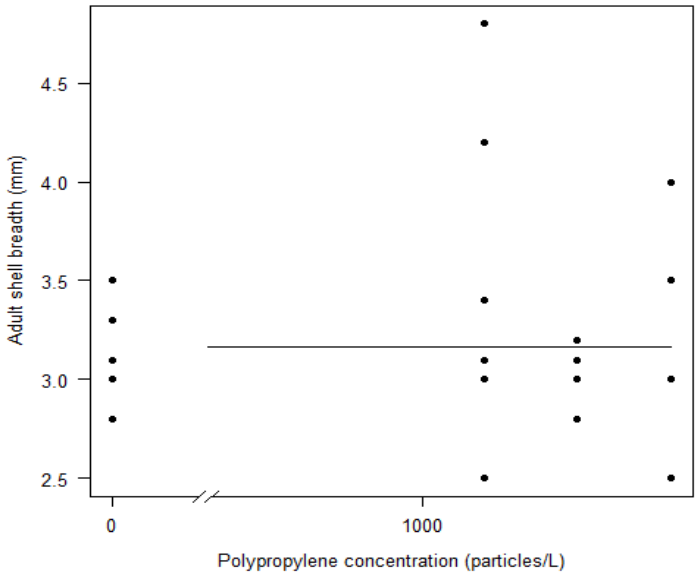



**Figure 3-4: Dose-response curve showing growth as shell breadth in adult snails in varying concentrations of polypropylene suspension.**

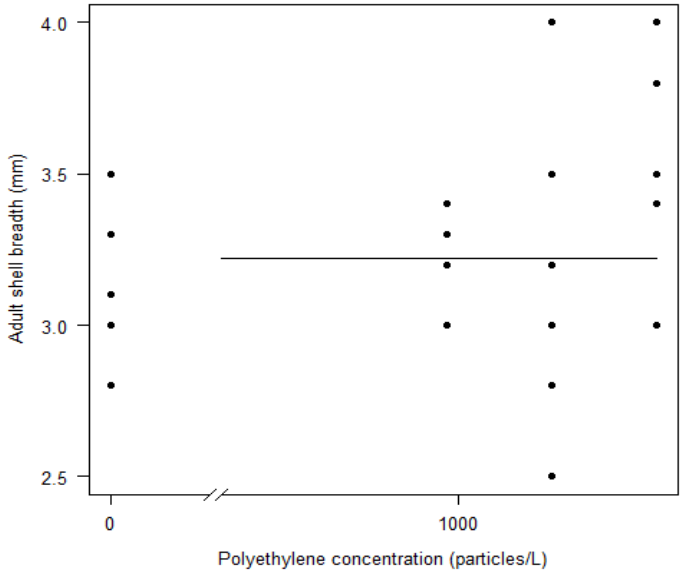



**Figure 3-5: Dose-response curve showing growth as shell breadth in adult snails in varying concentrations of polyethylene suspension.**

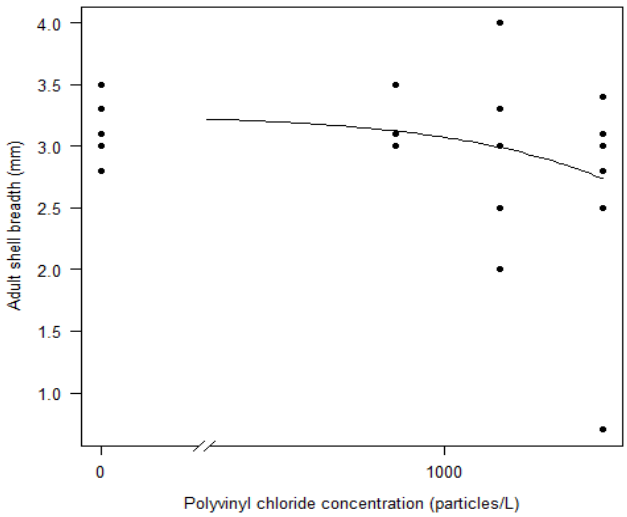



**Figure 3-6: Dose response curve showing growth as shell breadth in adult snails in varying concentrations of polyvinyl chloride suspension.**

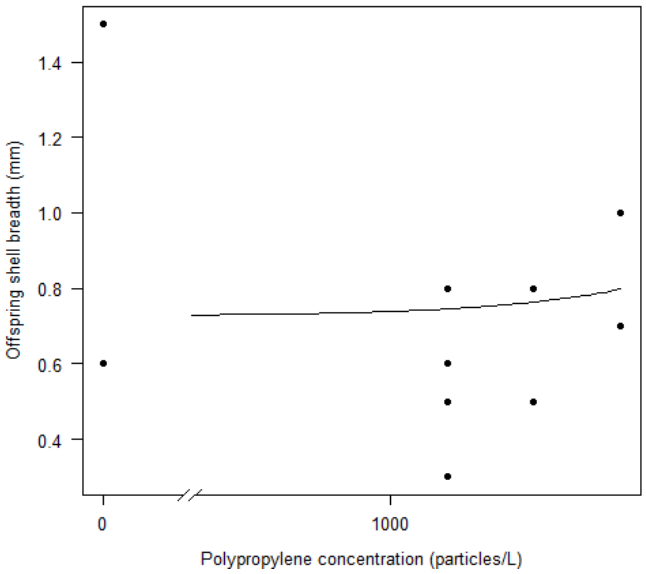
*3.3.3.3 Young snail growth test*

Figure 3-7A shows the growth response in young snails to varying doses of polypropylene. This makes the results from this assessment directly comparable to Figure 3-7A which is the same test on adult snails. As in Figure 3-7A, there is no clear response to varying levels of polypropylene. The curve fitting algorithm could only resolve one out of three curve parameters, and the lack-of-fitness test returned a p-value of 0.072.

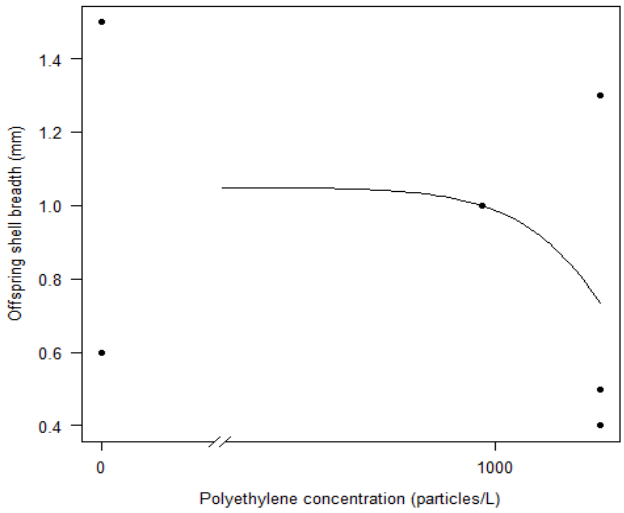
The growth response of young snails at varying levels of polyethylene are shown in Figure 3-7B. The fitted dose-response curve that is plotted in Figure 3-7B suggests that polyethylene at the highest levels assessed may cause a decrease in the growth of young snails. However, in common with most of the results presented here, the statistical significance of the fitted line's parameters is low, and no firm conclusions can be drawn about the negative effects of polyethylene exposure at the levels assessed. It is possible that a repeated experiment, or an experiment with greater levels of polyethylene, might produce statistically significant results.

The growth response of young snails exposed to varying polyvinyl chloride particles in suspension, and the fitted dose-response curve, are shown in Figure 3-7C
Figure 3-7: Dose-response curve showing growth as shell breadth in young snails in varying concentrations of polypropylene (A), . The dose-response curve suggests that growth is slowed at higher levels in young snails to a greater extent than in adult snails. However, variation in the data remains high, and the fitted curve cannot be said to show the polyvinyl chloride response significantly. The curve fitting process only produced one statistically significant parameter (out of three), and the standard error around the $EC_{50}$ and $EC_{10}$ estimates are high. Statistical significance of estimates might be improved by more significant replication, testing to higher microplastic concentrations, or both.

A




B




C

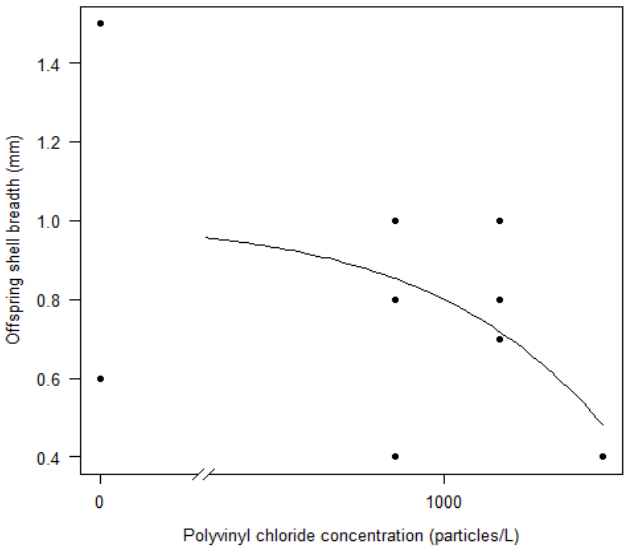



**Figure 3-7: Dose-response curve showing growth as shell breadth in young snails in varying concentrations of polypropylene (A), polyethylene (B) and polyvinyl chloride (C) suspension.**

### 3.3.4 Effect of different microplastic types and concentrations on Tilapia

#### *3.3.4.1 Tilapia growth-length*

Figure 3-8 shows the effect of polypropylene particles on the growth of *Tilapia* as measured by body length after 21 days' exposure. The fitted dose-response curve suggests a slight decrease in growth over the concentrations assessed. Again, variation in data was high, and firm statistically supported conclusions cannot be drawn about the curve. Curve fitting processes were able to produce two out of three curve parameters that were statistically supported; however, lack of fitness testing did not suggest a significantly bad fit of the model (p=0.588). Figure 3-9 shows variation in the length of juvenile *Tilapia* after 21 days of exposure to varying levels of polyethylene. As with many examples, the fitted curve suggests a slight decrease in growth after exposure to polyethylene. However, variation in data is high enough that little can be reliably inferred from the fitted curve. The curve fitting algorithm was only able to find one out of three curve parameters with statistical support, and as a result, there is insufficient clear support for a decrease in the growth of *Tilapia* after exposure to polyethylene particles. Figure 3-10 shows the body length of juvenile *Tilapia* after exposure to varying levels of polyvinyl chloride for 21 days. The data and the fitted curve in Figure 3-10 do not suggest any response to the microplastic at the levels tested. The curve fitting algorithm was only able to fit one curve parameter out of three.

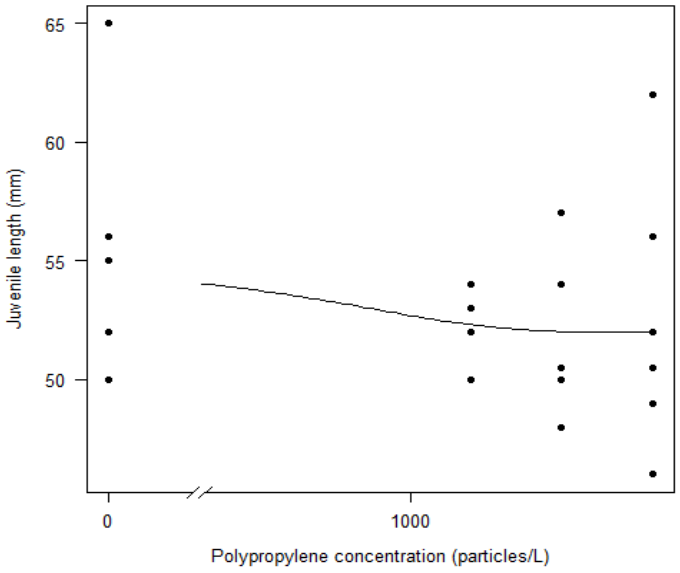



**Figure 3-8: Dose-response curve showing growth as body length in juvenile *Tilapia* after 21 days in varying concentrations of polypropylene suspension.**

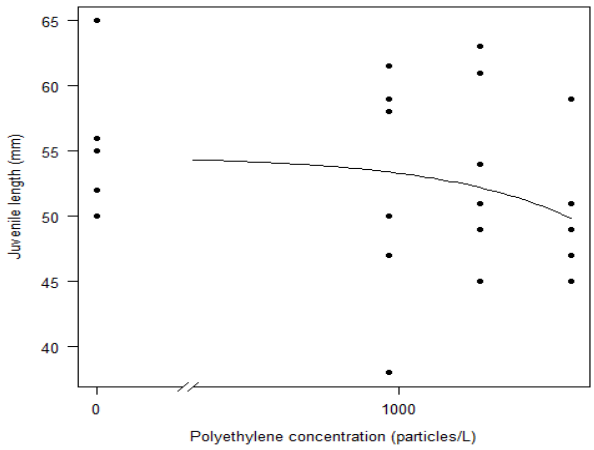



**Figure 3-9: Dose-response curve showing growth as body length in juvenile *Tilapia* after 21 days in varying concentrations of polyethylene suspension.**

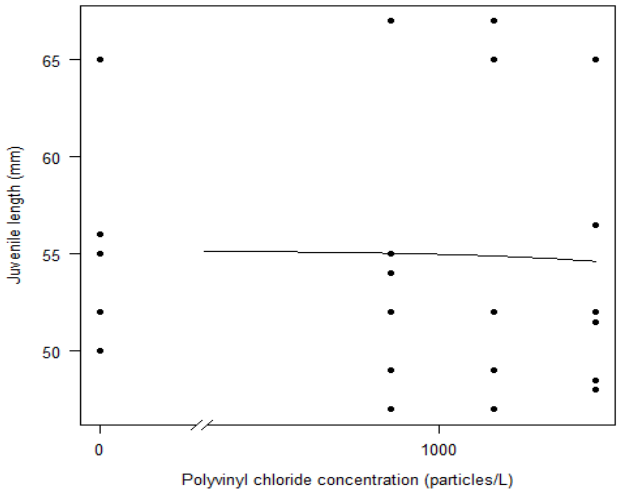
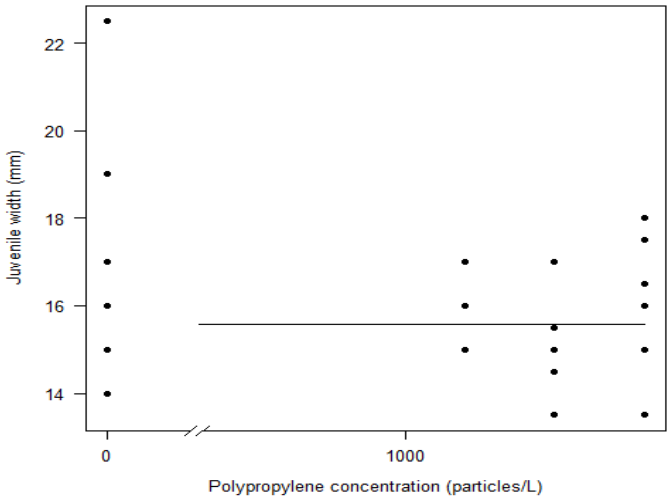



**Figure 3-10: Dose-response curve showing growth as body length in juvenile *Tilapia* after 21 days in varying concentrations of polyvinyl chloride suspension.**

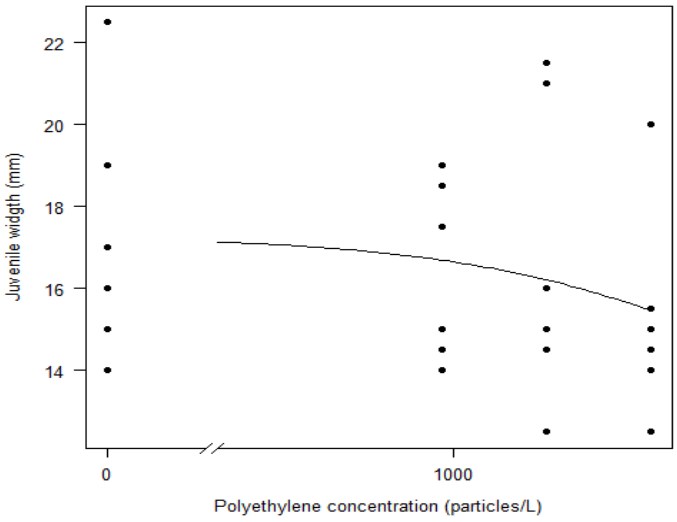
### *3.3.4.2 Tilapia growth-width*

Figure 3-11 shows growth as body width after 21 days of exposure to varying concentrations of polypropylene, polyethylene and polyvinyl chloride particles. The data and fitted curve for polypropylene (Figure 3-11A) suggests that no response to polypropylene in this experiment could be identified. The curve fitting process made statistically significant estimates of all three curve parameters. The clearer results, and the relatively low standard error on $EC_x$ estimations, seems to be largely a function of low, unchanging variation across exposures rather than a clear response to different microplastic exposures. Figure 3-11B shows juvenile Tilapia's body width after 21 days' exposure to polyethylene particles. The fitted growth curve suggests a decrease in growth in higher microplastic exposures, but statistics do not clearly support this. The variation in data is apparent and the curve fitting procedure one fits one of three curve parameters. Similarly, Figure 3-11C shows juvenile *Tilapia* body width after exposure to varying levels of polyvinyl chloride. The fitted dose-response curve suggests a greater growth rate in higher microplastic levels, but this is not supported statistically. Variability in raw data is apparent in the plot. The curve fitting procedure could not estimate any of the three curve parameters with statistical support, and a lack of fit test indicated that the curve did not describe the data well ($p<0.001$). As a result, one cannot identify any statistically supported response of *Tilapia* growth as width to polyvinyl chloride.

A




B




C

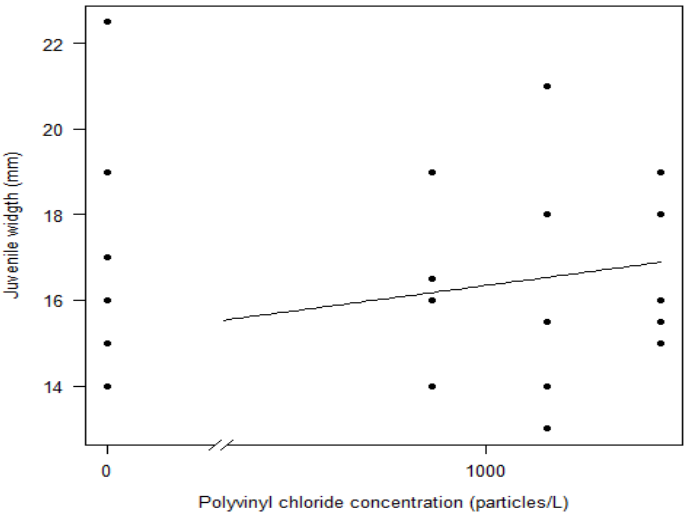



**Figure 3-11: Dose-response curve showing growth as body width in juvenile *Tilapia* after 21 days in varying concentrations of polypropylene (A), polyethylene (B) and polyvinyl chloride (C) suspension.**

### *3.3.4.3 Tilapia growth-mass*

Figure 3-12 shows *Tilapia* mass after 21 days' exposure to various concentrations of polypropylene particles. Assessment of the raw data does not suggest any significant response to the microplastic particles, and the fitted response curve likewise does not indicate a response to different polypropylene levels. The curve fitting process established a statistically significant estimate of one of three curve parameters, and the lack of fit test did not indicate a bad fit of the curve to the data (p=0.271). Figure 3-13 shows variations in juvenile *Tilapia* body mass after 21 days of exposure to varying levels of polyethylene.

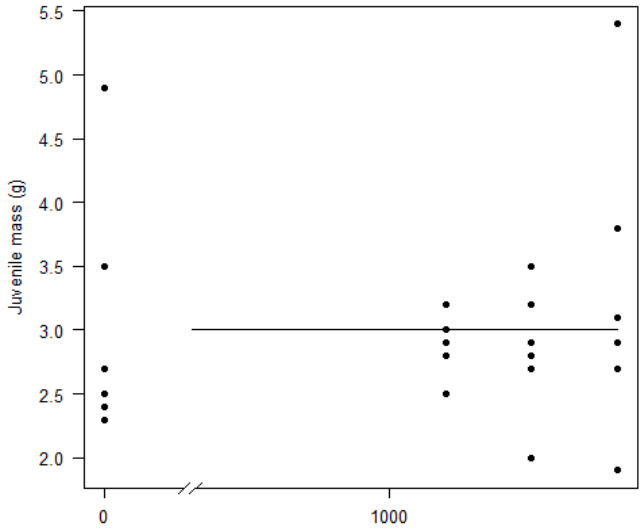



**Figure 3-12: Dose-response curve showing growth as body mass in juvenile *Tilapia* after 21 days in varying concentrations of polypropylene suspension.**

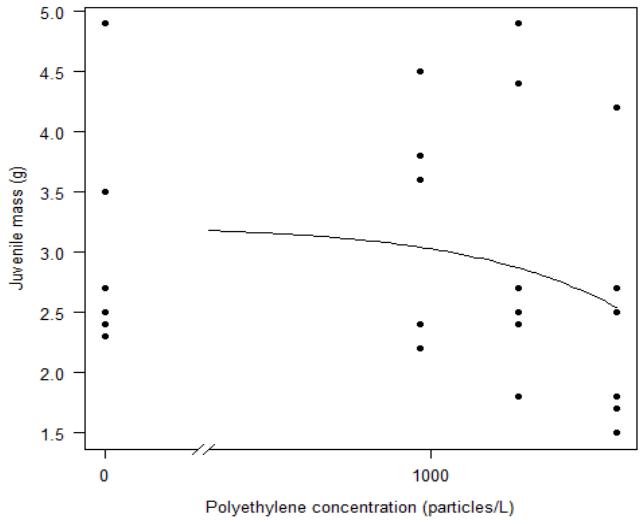



**Figure 3-13: Dose-response curve showing growth as body mass in juvenile *Tilapia* after 21 days in varying concentrations of polyethylene suspension.**

The data in the plot are very variable, and the fitted dose-response curve suggests a decrease in mass at the highest microplastic levels. However, the curve fitting process could only reliably fit one of three curve parameters, and as a result, the model is not well supported. Figure 3-14 show the body mass of juvenile *Tilapia* after exposure to various levels of polyvinyl chloride particles for 21 days. As in many other plots in this report, the fitted dose-response curve suggests a decrease in growth in higher levels of microplastic particles, a conclusion that is at best marginally supported by data. The curve fitting process only estimated one of three curve parameters with statistical support. As a result, the curve does not have strong statistical support.

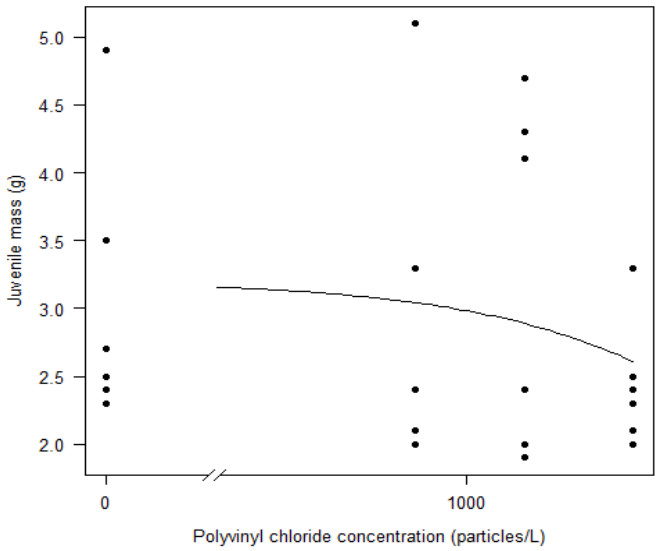



**Figure 3-14: Dose-response curve showing growth as body mass in juvenile *Tilapia* after 21 days in varying concentrations of polyvinyl chloride suspension.**

### 3.3.5 Ingestion and egestion of microplastic particles by the freshwater fish

Figure 3-15 shows the number of microplastic particles egested over 21 days of exposure to differing levels of polypropylene. The data and the fitted dose-response curve indicate no consistent change in microplastic egestion with the concentration of polypropylene in the exposure. The curve fitting procedure could not assign any of the three curve parameters at a statistically significant level. Simple log-linear regression is likewise unable to generate a statistically significant fit (p=0.546).

Figure 3-16 shows changes in microplastic particle egestion by juvenile *Tilapia* in various levels of polyethylene after 21 days' exposure. Both the data and the fitted response curve suggest increased egestion with greater microplastic exposure. However, the curve fitting procedure fitted none of the three curve parameters to a statistically significant level. A simple log-linear regression did find a significant relation between polyethylene levels and particle egestion (p<0.001).

Figure 3-17 plots the accumulated microplastic particle egestion by juvenile *Tilapia* in differing levels of suspended polyvinyl chloride particles. The fitted curve suggests an increase in egestion with increased polyvinyl chloride exposure. A simple linear curve fitted returned a significant response to changes in polyvinyl chloride levels (p<0.001). The final test that was assessed was the relation between microplastic exposure and the number of plastic fibers that were egested by juvenile *Tilapia* over a 21-day exposure. Dose-response modeling of results from this test found a significant response at times,

and it is therefore not surprising that comparison of endpoints found that significant differences between microplastics were present ($p<0.001$). However, differing concentrations could not be statistically distinguished. The number of pellets egested was greater for polyethylene than for polypropylene ($p=0.002$). The number of fibers egested owing to polyvinyl chloride exposure intermediate between these extremes and not statistically distinguishable from these extremes either. Again unsurprisingly, the statistical model adopted is distinct from the null model ($p=0.001$), indicating that microplastic types are significant for this endpoint.

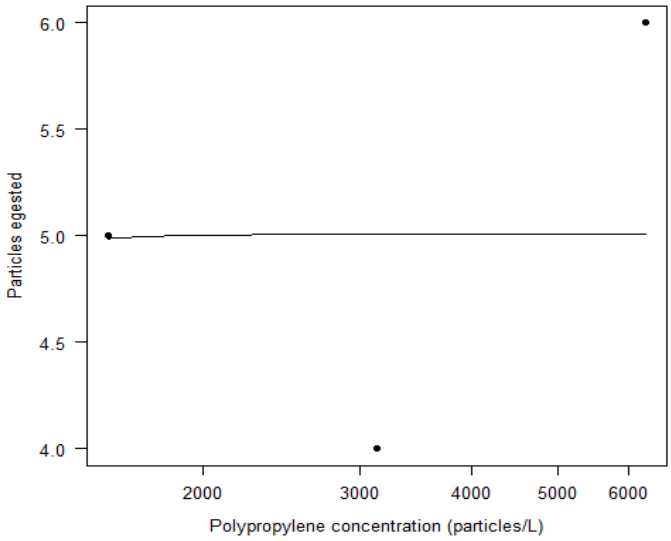



**Figure 3-15: Dose-response curve showing particles egested over 21 days by juvenile *Tilapia* in varying concentrations of polypropylene suspension.**

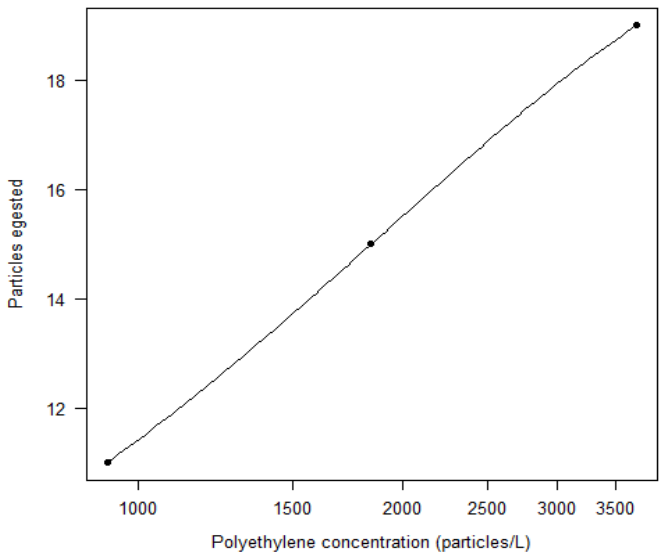



**Figure 3-16: Dose-response curve showing particles egested over 21 days by juvenile *Tilapia* in varying concentrations of polyethylene suspension.**

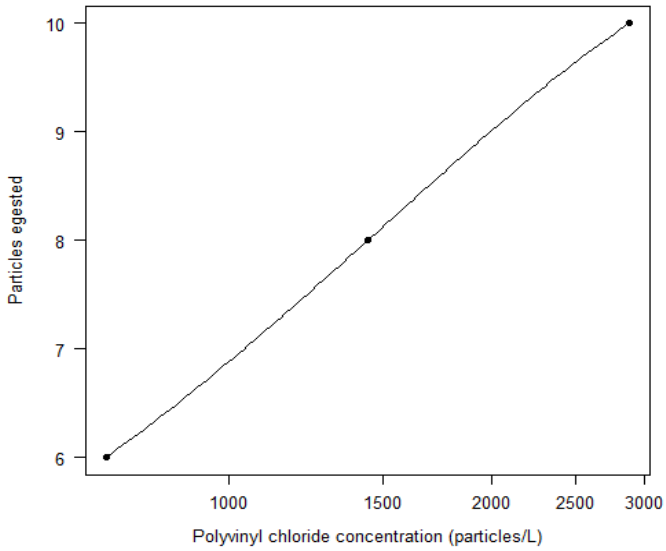



**Figure 3-17: Dose-response curve showing particles egested over 21 days by juvenile *Tilapia* in varying concentrations of polyvinyl chloride suspension.**

## 3.4 SUMMARY OF FINDINGS

A significant conclusion that can be drawn from the data and statistics presented here is that microplastic particles had a little detectable impact on *M. tuberculata* or *T. sparrmanii*, and this remained the case regardless of whether growth or reproduction-related endpoints were selected. The only statistically significant link between microplastic levels and the biotic response was a link between the amount of microplastic present and the number of pellets egested by *T. sparrmanii*. This result indicate that increased levels of microplastic increased microplastic avoidance behaviour. However, although this response was significant, this was not associated with growth changes or reduced reproductive success. An important point about these results is that the levels of microplastic assessed in these tests, in general, exceeded that found in the environment in South Africa and most other places that have been reported on (e.g. Li *et al*., 2018; Li *et al*., 2020; Bouwman *et al*., 2018). As no clear changes to growth rates of reproductive success were found at the levels of microplastic tested, the implication is that the negative effects of microplastic levels on the growth and reproduction of freshwater fauna may be limited. This is following other reporting, which noted that, although impacts resulting from exposure to microplastics have been found, negative effects were associated with environmentally unrealistic levels of microplastics (SAPEA, 2019; Triebskorn *et al*., 2019; GESAMP, 2016; Tang, 2017; Lenz *et al*., 2016).

These conclusions come with a *caveat*. Inspection of the dose-response curves reveals that the fitted curve often indicates a negative response at higher exposure levels. Because of the variation in the data, these responses were not statistically significant in any particular test. However, non-significant responses were relatively common across the tests, and these responses were all negative, without the positive responses that would be anticipated if these results were purely random. As a result, the possibility exists that repeating these tests with far greater replication, or using laboratory or statistical methods to control variation further, may lead to more significant responses. However, an inspection of the dose-response curves indicates that these non-significant responses only occur at high levels of microplastic, which may not be environmentally realistic.

Another point to consider is that the test taxa reported here do not include any feeding strategy to maximize microplastic uptake. The inclusion of a filter-feeder as a test taxon might have revealed a greater response to microplastics provided that uptake of these was high due to feeding processes (e.g. Qu *et al*., 2018; Berglund *et al*., 2019).

The exposures reported here were undertaken to test the impacts owing to the physical uptake of or exposure to microplastic particles. This may relate to chemical stressors such as plasticisers, dyes, *et cetera* leaching from the microplastic particles. The lack of significant impact resulting from microplastic particles does not suggest a significant chemical impact. The ratio of different shapes varied between the different microplastics, and shape and size have been proposed to modify the impacts caused by microplastics (e.g. Burns and Boxall, 2018; Wright *et al*., 2018). The lack of any clear difference between the different microplastics indicates that at the quantities tested, the greater proportion of fibres in polyethylene, and irregular shapes in the others, had little or no detectable effect.

The findings presented here show that the responses of test taxa to environmentally realistic levels of microplastics were essentially minimal. Tests that used survival, growth and replication as endpoints mostly showed no clear response to the tested microplastics. The only apparent response to microplastics was increased microplastic pellet egestion at higher microplastic concentrations, which does not indicate a toxic effect *per se* but rather a microplastic clearing mechanism. That no responses were found during the test exposure does not rule out other potential impacts owing to, for example, long term plastic accumulation in the gut and consequent feeding reduction. However, although microplastic responses were limited, supporting the hypothesis that an element of potential microplastic toxicity is due to plasticisers. However, a dose-response relationship was more commonly associated with some endpoints related to reproduction, and no real change in growth in response to plasticiser concentrations was found. Some cellular level response was found, though high variation in results made this difficult to quantify.

# CHAPTER 4: ASSESSING THE TOXICITY OF PLASTICISERS ON SELECTED FRESHWATER ORGANISMS IN RIVERINE SYSTEMS IN SOUTH AFRICA

## 4.1 INTRODUCTION

Plastic pollution is one of the fastest-growing environmental challenges of our time. It threatens our ability to implement most, if not all, of the United Nations Sustainable Development Goals, including clean water and sanitation (Abduro Ogo *et al*., 2022; Zhang *et al*., 2022). Continued overproduction by the plastics industry, combined with widespread consumer use and overconsumption, has resulted in the full plastic materials being a significant cause of water pollution (Kurniawan *et al*., 2021). This has resulted in the widespread release and transfer of thousands of toxic chemicals into the environment and organisms (Zhang *et al*., 2022). In the last decade, plasticisers released from plastics during plastic production and in the environment have become a major environmental pollutant, affecting both humans and organisms (Li *et al*., 2022) microplastics 2022. Plasticisers, such as phthalates, Bisphenol-A and Calcium stearate, are added to plastics (e.g. polyvinyl chloride) to make them more flexible and durable; these plastics generally contain up to 50% plasticisers by weight (Nshimiyimana *et al*., 2020). Phthalates are also additives in fragrances, paints and sealants, cardboard, adhesives, lubricants, ink, pesticide and herbicides (Kanaujiya *et al*., 2022). However, phthalates are not physically bound to the plastic polymer and can easily leach into the environment during manufacture, use, and disposal (Boonnorat *et al*., 2016). Leaching of plasticisers from plastic polymers has commonly been noted in a process that introduces the plasticiser to the plastic's environment and embrittlement (Gugliandolo *et al*., 2020; Wang *et al*., 2014). Plasticisers can leach when they have not formed chemical bonds with the polymer that they are dispersed in (Kastner *et al*., 2012). The introduction of plasticisers to the environment may cause a chemical threat. This would apply regardless of whether the danger is caused by the plasticiser itself or its breakdown products.

Plasticisers in the environment can affect organisms and humans through various pathways such as ingestion, inhalation, skin absorption/contact and intravenous injection (Tuan Tran *et al*., 2022). For example, freshwater organisms can be easily exposed to plasticisers through feeding in water, with suspension feeders potentially more affected. On the other hand, human exposure to PAEs can happen through various pathways such as ingestion, inhalation, skin absorption/contact, and intravenous injection (Tran *et al*., 2022). Most plasticisers are regarded as disruptive endocrine chemicals (EDCs) and can cause substantial harm to humans' respiratory, reproductive, and endocrine systems (Sun *et al*., 2013; Wang *et al*., 2018). For example, many studies showed that PAEs toxicity leads to reproductive failure related to the testicular cell functions (Wang *et al*., 2014). In the freshwater environment, plasticisers can leach and persist at high concentrations, causing severe effects on the ecosystem and human health via the food chain Fromme *et al*. (2002). For example, Fromme *et al*. (2002) reported that phthalates detected in surface water were 22.7 mg $L^{-1}$, whereas the highest concentration of 288 mg $L^{-1}$ was found in the wastewater (Salaudeen *et al*., 2018). Effects associated with these compounds, such as reduced fertility, feminisation, reproductive organ abnormalities, or altered sexual behaviour, have been observed in mammals, fishes, benthonic organisms, etc. (Domínguez-Morueco *et al*., 2014).

Despite plasticisers being recognised to cause harmful effects on human and aquatic organisms and their continuous usage, much of the studies on plasticisers are on analysing their presence in water

bodies. Therefore, it is essential to investigate the toxicity of plasticisers used for plastic production to understand how and what concentrations they affect aquatic organisms. The few available studies are from other regions (e.g. Domínguez-Morueco *et al*., 2014; Lee *et al*., 2015; Wang *et al*., 2018), further limiting our knowledge of the effects of plasticisers on local and endemic organisms. Hence, this chapter aimed to investigate the toxicity of plasticisers (phthalates, Bisphenol-A and Calcium stearate) on selected freshwater organisms.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Plasticiser solution preparation

Test concentrations of the plasticisers dibutyl phthalate (DBP) and Bisphenol A (BPA) were obtained from the literature based on concentration obtained in the environment (Abdel-Tawwab and Hamed 2018; Edjere *et al*., 2016; Oehlmann *et al*., 2006, 2009). For calcium stearate (CAS), range-finding tests were used to determine exposure concentrations due to unavailable information on calcium stearate in freshwater. Because of low solubility in water, dibutyl phthalate was solubilized in equal quantities of acetone, and calcium stearate was solubilized using small quantities of EDTA. The concentrations tested ranged up to 0.002152 µL/l for dibutyl phthalate, 4 µg/l for Bisphenol A, and 5 mg./l for calcium stearate ((Table 4-1). The sole exception to this was the concentrations tested in the 96 hr lethality test, which used higher concentrations of plasticisers (see Table 4-2) to generate a toxicological result after a relatively short exposure.

**Table 4.1: Concentrations used in toxicological tests of various plasticisers. Control exposures used dechlorinated water. Dibutyl phthalate had a positive acetone control, and calcium stearate had a positive EDTA control.**

| **Plasticiser** | **Concentration gradient** | | | | | |
|---|---|---|---|---|---|---|
| Dibutyl phthalate (µL. $L^{-1}$) | 0 | 0.00014 | 0.00027 | 0.00054 | 0.00108 | 0.00215 |
| Bisphenol A (µg. $L^{-1}$) | 0 | 0.25 | 0.50 | 1.00 | 2.00 | 4.00 |
| Calcium stearate (mg. $L^{-1}$) | 0 | EDTA | 0.625 | 1.25 | 2.50 | 5.00 |

**Table 4.2: Concentrations of plasticisers used in 96 hr lethal testing. Control exposures used dechlorinated water. Dibutyl phthalate had a positive acetone control, and calcium stearate had a positive EDTA control.**

| **Plasticiser** | **Concentration gradient** | | | | | | |
|---|---|---|---|---|---|---|---|
| Dibutyl phthalate (mg. $L^{-1}$) | 0 | Acetone | 8 | 10 | 12 | 14 | 16 |
| Bisphenol A (µg. $L^{-1}$) | 0 | | | | | | |
| Calcium stearate (mg. $L^{-1}$) | 0 | EDTA | 100 | 200 | 300 | 400 | 500 |

### 4.2.2 Toxicological tests of reproduction and growth

The endpoints were monitored to assess the potential toxic effect of the plasticiser's calcium stearate, dibutyl phthalate and Bisphenol A on a range of test taxa. Table 4-3 shoes the endpoints selected to reflect the impact of the plasticisers on the survival, growth and reproduction in these taxa. The tests used adult and earlier life-history stages to determine whether plasticisers might have an impact

throughout an organism's life history. Short-term testing of taxa in the presence of solutions of various plasticisers used a standard protocol for all taxa. The protocol loosely follows the 96-hr fish lethality test presented as part of DEEEP (Slabbert, 2004). *Caridina nilotica* juveniles, or *Melanoides tuberculata*, or *Danio rerio* were placed into replicate plasticiser solutions and monitored daily to determine whether mortality had occurred until the test ended at 96 hours.

**Table 4.3: Endpoints selected for toxicological tests of reproduction and growth.**

| Test | Endpoint |
|---|---|
| Zebrafish mortality (96 hours) | Count of dead individuals |
| Snail mortality (96 hours) | Count of dead individuals |
| Shrimp mortality (96 hours) | Count of dead individuals |
| Zebrafish egg hatching test (5 days) | Count of hatched eggs (out of three) |
| Snail reproduction test (15 days) | Offspring produced per adult (two or five per replicate) |
| Snail reproduction test (21 days) | Offspring produced per adult (two or five per replicate) |
| Snail growth test-adult (15 days) | Individual adult shell length in mm. |
| Snail growth test-adult (21 days) | Individual adult shell length in mm. |
| Snail growth test-offspring (15 days) | Individual offspring shell length in mm. |
| Snail growth test-offspring (21 days) | Individual offspring shell length in mm. |
| Shrimp growth (21 days) | Length of juvenile in mm |
| Tilapia growth (21 days) | Length of juvenile in mm |

To assess the impact of plasticisers on fish reproduction and development, three eggs of *D. rerio* were placed into each of the replicate solutions and monitored for five days to determine success in hatching and development. Another test of the impact of plasticisers on reproduction and development was undertaken using the snail *M. tuberculata*. Groups of two to five *M. tuberculata* were placed in test solutions, and the production and growth of offspring were monitored for 21 days. Juvenile *C. nilotica* and *T. sparrmanii* were placed into each replicate of the plasticiser solutions and monitored for 21 days to determine the potential impact of the plasticisers on growth in these taxa. All test taxa were frozen at -20˚C at the end of each exposure to provide material for assessing enzyme systems function after plasticiser exposure as described in Section 3.2.3.

### 4.2.3 Stress enzymes

All test taxa used for toxicological tests of reproduction and growth were frozen at -20˚C at the end of each exposure to provide material for the assessment of enzyme systems function after plasticiser exposure. Sections 4.2.3.1-4.2.3.3 and Table 4-4 provide details of the enzyme functions assessed.

**Table 4.4: Endpoints selected for toxicological tests of enzymes stress.**

| Test | Endpoint |
|---|---|
| AChE levels (21 days) | Acetylcholinesterase activity per unit protein (units/mg) |
| LPx levels (21 days) | Lipid peroxidase per unit protein (nmol/mg) |

#### *4.2.3.1 Acetylcholinesterase assay*

Acetylcholinesterases are enzymes that hydrolyse the neurotransmitter acetylcholine to acetate and choline. Changes in acetylcholinesterase activity may result from exposure to chemical stressors including certain insecticides. Acetylcholinesterase activity was measured using a Sigma MAK119 kit. This assay is an optimised version of the Ellman method in which thiocholine, produced by acetylcholinesterase, reacts with 5,5-dithiobis(2-nitrobenzoic acid) to form a colorimetric (412 nm) product, proportional to the acetylcholinesterase activity present. One unit of acetylcholinesterase is the enzyme that catalyses the production of 1.0 millimole of thiocholine per minute at pH 7.5 at room temperature.

Samples were defrosted and brought to room temperature and then macerated and homogenised in 0.1 M phosphate buffer at pH 7.5 to extract the proteins present. The extracts were clarified by centrifuging them at 14000 rpm for 5 minutes. 100 µl of supernatant were collected for the acetylcholinesterase assay and analysed colorimetrically following kit instructions.

#### *4.2.3.2 Lipid peroxidase assay*

Lipid peroxidation is the degradation of lipids that occurs as a result of oxidative damage and is a useful marker for oxidative stress. Polyunsaturated lipids are susceptible to an oxidative attack, typically by reactive oxygen species, resulting in a well-defined chain reaction with the production of end products such as malondialdehyde. Lipid peroxidase activity was assessed using a Sigma MAK085 kit. In this kit, lipid peroxidation is determined by the reaction of malondialdehyde with thiobarbituric acid to form a colorimetric product, proportional to the MDA present. 10 mg samples were homogenised on ice in300 µL of malondialdehyde lysis buffer containing 3 µL of butylated hydroxytoluene. Samples were then centrifuged at 13000 g for 10 mins, and then 200 µl of supernatant were taken for further colorimetric analysis of lipid peroxidase following the kit's instructions.

## 4.3 RESULTS

### 4.3.1 Sensitivity of the selected aquatic organisms to different types an concentrations of plasticisers

The fish egg hatching test appeared to show some inhibition in response to higher concentrations of two plasticisers. The results of an analysis of deviance comparison of endpoints from the *D. rerio* 5-day egg hatching test found a significant difference in the results due to the plasticiser tested (p=0.020), but not to the plasticiser concentration or the interaction between the two. Comparison of the derived model with the null model revealed that the two could not be statistically distinguished. The detectable changes due to the treatments were small compared to other variations in egg hatching rates.

The 21-day snail reproduction test was the only test of those assessed for the current report to elicit a standard S-shaped log-logistic curve when applied with Bisphenol-A. A comparison of endpoints assessed was only able to compare the points for Bisphenol A and calcium stearate, as the dibutyl phthalate test was terminated owing to the Covid lockdown before it reached data 21. An analysis of deviance found that the interaction between plasticiser type and concentration was statistically significant (p=0.050), but that either plasticiser or concentration alone was not significant. However, the model was not significantly different from a null model (p=0.082). The plasticiser type and concentration did not explain a large amount of the variation in reproductive rates found.

Owing to the lack of day 21 dibutyl phthalate results, the day 15 results for all plasticisers was also compared statistically. Here neither plasticiser type nor concentration was significantly linked to day 15 reproductive rates (p=0.094 and p=0.087, respectively), and the model was not significantly different to the null model. The significance levels for plasticiser type and concentration indicate that it is possible that increasing the replication or finding and controlling other potential sources of variation might lead to a significant result.

Snail shell size (measured as length) was compared between the 15 and 21-day exposures to plasticisers that also assessed snail reproduction. This should indicate the potential of the plasticisers to limit growth in both adult and juvenile snails. While adult shell length may be affected by the condition of the adult at the start of the test, all juvenile growth takes place while exposed to plasticisers and if these limit growth in *M. tuberculate*, it should be apparent from the juvenile growth response. As in the snail reproduction test results, data from day 21 for dibutyl phthalate exposure is unavailable, and the day 15 results are used instead.

Day 21 adult shell lengths ($p<0.001$), day 21 juvenile shell lengths ($p=0.049$), and day 15 adult shell lengths ($p<0.001$) all showed a statistically significant response to the different plasticisers that were assessed. In some ways, this is a surprising result, as juveniles might be expected to be more sensitive as they spend their entire life exposed to plasticisers. Also, the dose-response curve fits revealed a lot of variation in data and no clear response to increasing concentrations of plasticisers. In the day 21 results, adults in Bisphenol A were more significant than those in calcium stearate, while juveniles in calcium stearate were larger than those in Bisphenol-A. In the day 15 results, the adults in calcium stearate were longer than those in dibutyl phthalate. There was no clear size difference between the juveniles.

The growth of juvenile *T. sparmanii*, as expressed by a length after exposure to plasticisers for 21 days, was not significantly changed by any of the plasticisers ($p=0.647$). However, there was a detectable link between plasticisers and growth of *T. sparrmanii* measured as breadth (0.033). Similarly to fish length, the development of the freshwater shrimp *C. nilotica* showed no differential growth patterns when exposed to plasticisers for 21 days ($p=0.121$).

### 4.3.2 Assessing the toxicity effects of different plasticiser concentrations – dose-response relations

Dose-response curves for the response of all test taxa to increasing exposure to the plasticisers dibutyl phthalate (DBP), Bisphenol A and calcium stearate are presented below. The $EC_{50}$ from these curves are in Table .5, while $EC_{10}$ data are in Table 4-5. These estimates assess the response of snails, fish and shrimp to the plasticisers. Tests undertaken assess the response of test taxa in terms of mortality, other endpoints that consider impacts on growth and reproduction, and the responses of adults, juveniles, and even eggs.

**Table 4.5: $EC_{50}$ data from toxicity tests using the plasticisers calcium stearate (CAS), dibutyl phthalate (DBP) and Bisphenol A (BPA). $EC_{50}$ data are presented with standard errors. NA indicates either that there was no detectable dose-response, that $EC_{50}$ estimates were negative or that the curve-fitting algorithm failed to fit a curve to the data.**

| Test | CAS (mg/L) | BPA (µ/L) | DBP (µL/L) |
|---|---|---|---|
| Zebrafish egg hatching test (5 days) | 1.55±0.33 | 38.17±519.04 | NA |
| Tilapia growth juvenile length (21 days) | NA | NA | NA |
| Tilapia growth juvenile breadth (21 days) | 2.29±5.65 | NA | 10.7±113.9 |
| Shrimp growth (21 days) | NA | NA | NA |
| Snail reproduction test (15 days) | NA | 0.75±0.74 | 0.09±2.29 |
| Snail reproduction test (21 days) | NA | 0.61±0.64 | |
| Snail growth test-adult (15 days) | | | 9.59±22.13 |
| Snail growth test-adult (21 days) | NA | 7.45±11.39 | |
| Snail growth test-offspring (15 days) | | | 0.13±2.55 |
| Snail growth test-offspring (21 days) | 0.022±14.30 | NA | |
| Shrimp AChE levels (21 days) | 61.1 ± 493.4 | 320.5 ±8195.5 | 48.5 ± 829.1 |
| Shrimp LPx levels (21 days) | 6.0 ± 119.8 | 0.7 ± 84.7 | 91.1 ± 540.2 |
| Fish AChE levels (21 days) | 0.928 ± 10.32 | 0.324 ± 0.139 | 0.881 ± 0.136 |
| Fish LPx levels (21 days) | 0.147 ± 4.00 | 20.5 ± 271 | 0.105 ± 0.272 |

**Table 4.6: $EC_{10}$ data from toxicity tests using the plasticisers calcium stearate (CAS), dibutyl phthalate (DBP) and Bisphenol A (BPA). $EC_{10}$ data are presented with standard errors. NA indicates either that there was no detectable dose-response, that $EC_{10}$ estimates were negative, or that the curve-fitting algorithm failed to fit a curve to the data.**

| Test | CAS (mg/L) | BPA (µg/L) | DBP (µL/L) |
|---|---|---|---|
| Zebrafish egg hatching test (5 days) | 0.95±1.29 | NA | NA |
| Tilapia growth juvenile length (21 days) | NA | NA | NA |
| Tilapia growth juvenile breadth (21 days) | 1.83±1.84 | NA | NA |
| Shrimp growth (21 days) | NA | NA | |
| Snail reproduction test (15 days) | NA | 0.16±0.32 | 0.02±0.44 |
| Snail reproduction test (21 days) | NA | 0.11±0.24 | |
| Snail growth test-adult (15 days) | | | NA |
| Snail growth test-adult (21 days) | NA | 4.07±6.16 | |
| Snail growth test-offspring (15 days) | | | 0.04±0.71 |
| Snail growth test-offspring (21 days) | 0.003±2.17 | NA | |
| Shrimp AChE levels (21 days) | 9.3 ±75.0 | 0.0015 ± 0.073 | 4.5 ±75.3 |
| Shrimp LPx levels (21 days) | 2.3 ±34.3 | NA | 10.1 ± 60.0 |
| Fish AChE levels (21 days) | 0.004 ± 5160 | 0.046 ± 0.044 | 0.368 ± 0.118 |
| Fish LPx levels (21 days) | 0.00006 ± 0.001 | 6.25 ± 27.0 | 0.012 ±0.030 |

The effect concentrations presented in Table 4-4 and Table 4-5 are often associated with relatively high standard errors, resulting from the high variations in the observed data, which illustrates that the $EC_x$ estimates are not always strongly statistically supported. Likewise, both tables contain a number of instances where, owing either to observed variation, a lack of response, or both, models could not be fitted and effect concentrations estimated. While failure to fit a dose-response model to plasticiser exposure was common for most endpoints tested, it is of note that enzyme systems assessed provided data that commonly allowed for estimation of an effect concentration, albeit with standard errors that remained high. After these tests, tests that assessed snail reproduction and the growth of those offspring were most successful in returning $EC_x$ estimates.

### 4.3.3 Effect of different plasticisers on Zebrafish-Fish egg hatching test

The response of fish eggs to dibutyl phthalate is presented in Figure 4-1. Although variation in results is high, the fitted curve suggests that the response of *D. rerio* eggs to dibutyl phthalate is absent at lower concentrations tested, and it seem that responses may start to occur at concentrations above 0.001 µL/L. The variation in response across a range of concentrations resulted in no reliable $EC_{50}$ being available from the curve. The response of fish eggs to Bisphenol A is presented in Figure 5-2. Within the tested range of concentrations, detectable responses are minor to absent. The standard error produced for the $EC_{50}$ estimate indicates that the estimate is not reliable. The response of fish eggs to calcium stearate is presented in Figure 5-3. There was no response to calcium stearate levels up to approximately 1 mg/L, but the rate of success in hatching decreased
sharply after that.

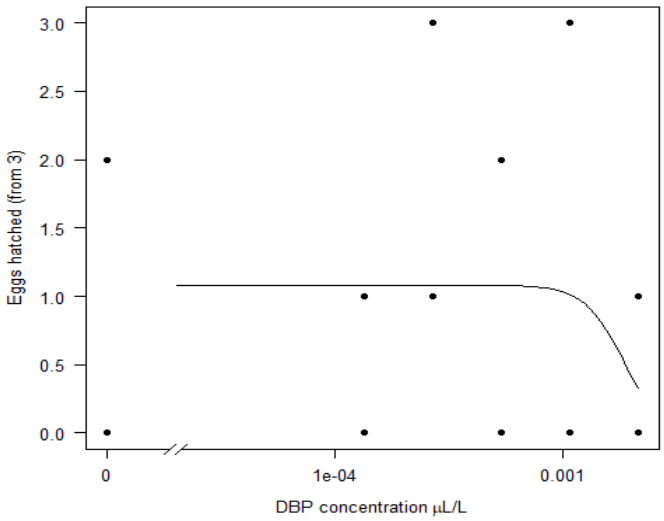



**Figure 4-1: Dose-response curve showing the number of eggs of D. rerio successfully hatching from three test eggs in a range of dibutyl phthalate solutions.**

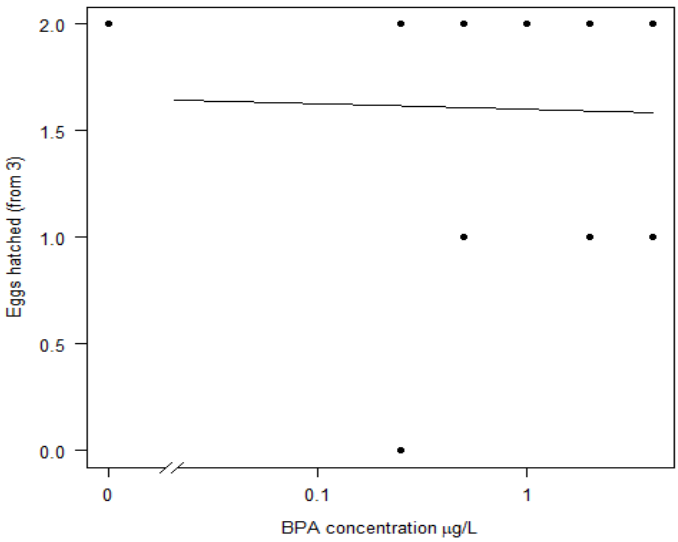



**Figure 4-2: Dose-response curve showing the number of eggs of D. rerio successfully hatching from three test eggs in a range of Bisphenol A solutions.**

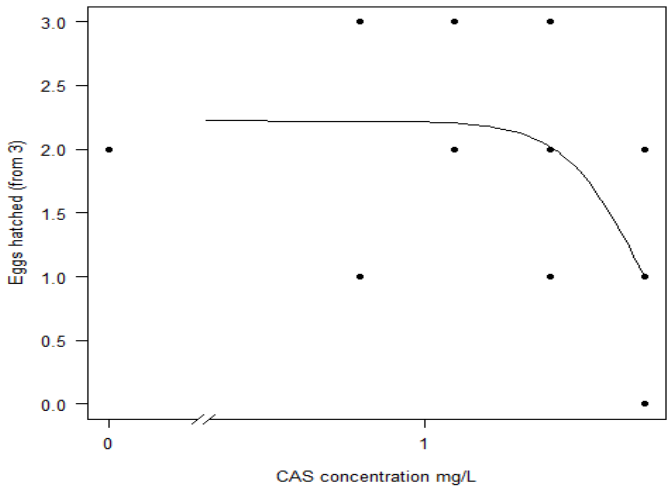



**Figure 4-3: Dose-response curve showing the number of eggs of D. rerio successfully hatching from three test eggs in a range of calcium stearate solutions.**

### 4.3.4 Effect of different plasticisers on snails

#### *4.3.4.1 Snail reproduction test*

Reproductive success in snails exposed for 15 days to a range of concentrations of dibutyl phthalate is presented in Figure 4-4. The experiment was terminated before it reached its 21-day endpoint, so the day 15 results are presented as an approximation. After 15 days at the range of concentrations tested, no clear response to dibutyl phthalate was detected. Variation in results was fairly high, as is apparent in Figure 4-5. Reproductive success in snails exposed for 21 days to a range of concentrations of Bisphenol A is presented in Figure 4-5. The curve shows a clear response to Bisphenol A, and reproductive success is decreased from approximately 1.5 offspring per adult snail in control solutions without Bisphenol A to approximately 0.2 offspring per adult snail in the higher concentrations. The curve fitting algorithm could not converge on a fit for snail reproduction after 21 days exposed to calcium

stearate solutions. As an approximation, reproductive success in snails exposed for 15 days is presented in Figure 4-6. These data suggest a slight decrease in reproductive success with increasing calcium stearate, but this conclusion is tentative due to variation in response. Inspection of day 21 data does not indicate any clear decrease in reproductive success with increasing calcium stearate.

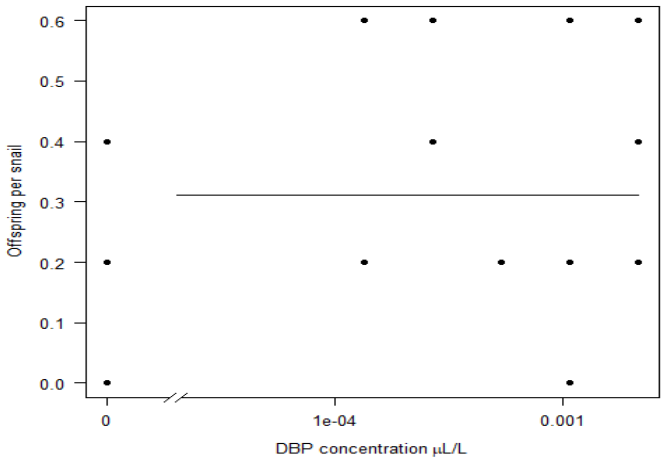



**Figure 4-4: Dose-response curve showing the number of offspring per adult snail of M. tuberculata successfully produced in a range of dibutyl phthalate solutions after 15 days.**

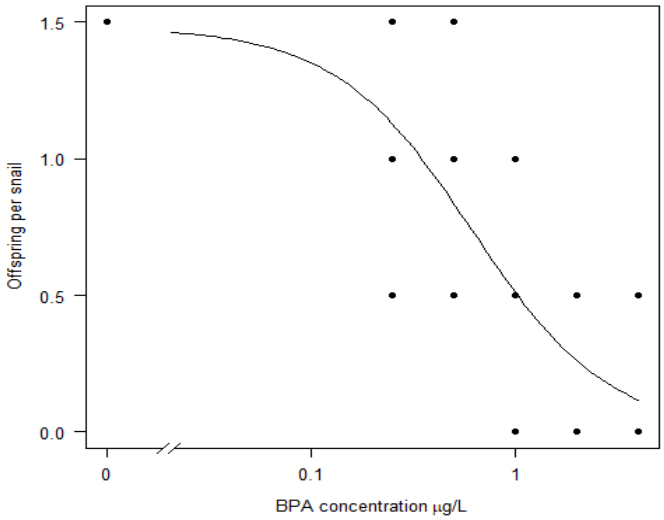



**Figure 4-5: Dose-response curve showing the number of offspring per adult snail of M. tuberculata successfully produced in a range of Bisphenol A solutions after 21 days.**

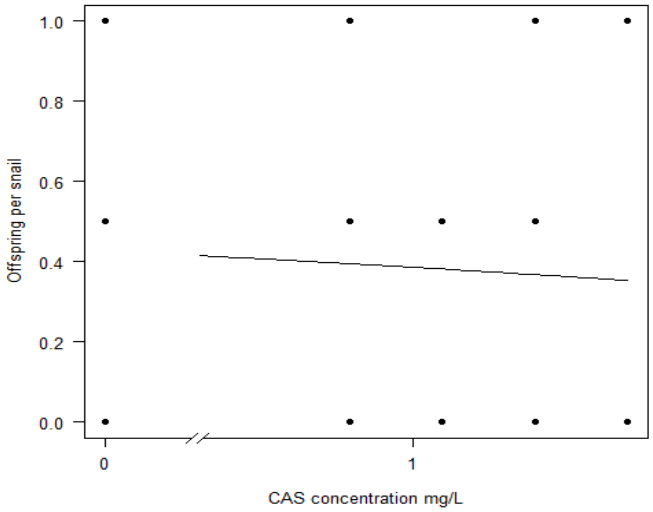



**Figure 4-6: Dose-response curve showing the number of offspring per adult snail of M. tuberculata successfully produced in a range of calcium stearate solutions after 15 days.**

#### *4.3.4.2 Adult snail growth test*

The exposure of snails to dibutyl phthalate was terminated before the intended 21-day endpoint, and the results from 15 days are presented here as an approximation. After 15 days of exposure to dibutyl phthalate, adult snail shell lengths are presented in Figure 4-7. A classic log-logistic growth response was not found in the results; however, a slight decrease in adult shell length was found. The shell length decrease was insufficient to half-shell length and returned an unambiguous EC50 value, possibly because adult snail shell size changes were mainly fixed before the experiment began.

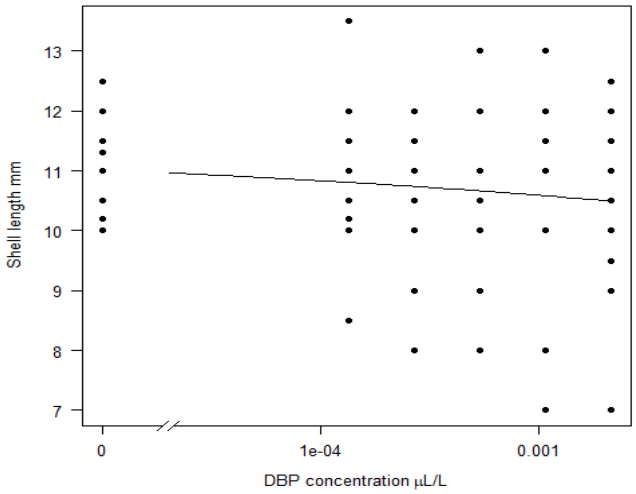



**Figure 4-7: Adult *M. tuberculata* shell length after exposure to dibutyl phthalate for 15 days.**

Juvenile *M. tuberculate* shell lengths after exposure to dibutyl phthalate for 15 days are presented in Figure 4-8. These juveniles were born during the experiment and have been exposed to dibutyl phthalate for their entire lives. Although adult *M. tuberculata* suggest some inhibition of growth by the plasticiser, the results from the exposure do not return the classic S-shaped log-logistic curve but rather indicate an exponential decrease in shell length with exposure dibutyl phthalate. However, two or three-

parameter exponential decay models could not return a better fit. It is unclear what to make of the data in Figure 4-8. Although the shell length of juveniles exposed to DBP was less than those in control, there is no clear response to increasing concentrations of DBP, and the data suggest a minor increase in shell length with increasing levels of DBP. The response of adult *M. tuberculata* shell lengths to increasing concentrations of Bisphenol A after 21 days are presented in Figure 4-9. Variability in data was high, with no clear change in adult shell length with increasing concentrations of Bisphenol A was found.

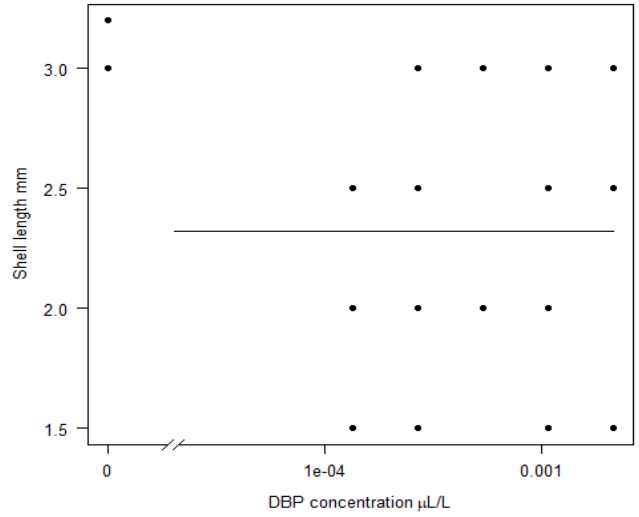



**Figure 4-8: Juvenile *M. tuberculata* shell length after exposure to dibutyl phthalate for 15 days.**

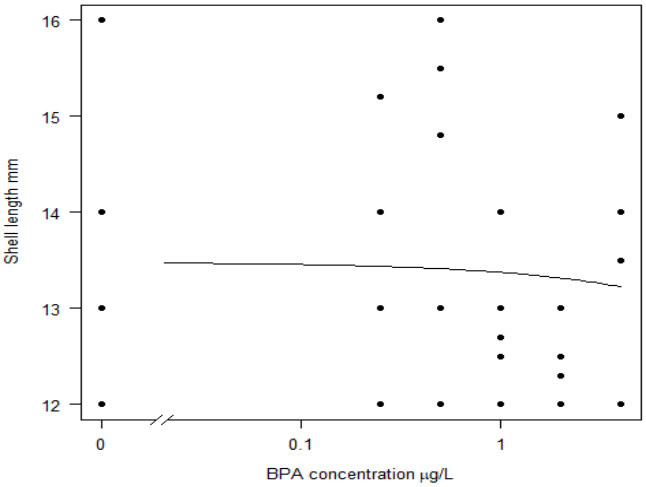



**Figure 4-9: Adult *M. tuberculata* shell length after exposure to Bisphenol A for 21 days.**

The response of juvenile *M. tuberculata* to exposure to increasing levels of Bisphenol A is presented in Figure 4-10. The plot does not show a log-logistic decrease of shell length with increasing levels of Bisphenol A, but the results suggest more of an exponential decrease of shell length on exposure to Bisphenol A. However, there is no clear change with increasing Bisphenol A levels, and curve-fitting routines failed to fit an exponential model to the data. The shell length of adult *M. tuberculata* exposed to calcium stearate for 21 days is presented in Figure 4-11. No change in shell length with increasing levels of calcium stearate was detected. The response of juvenile *M. tuberculata* shell length to calcium stearate after 21 days is presented in Figure 4-12.

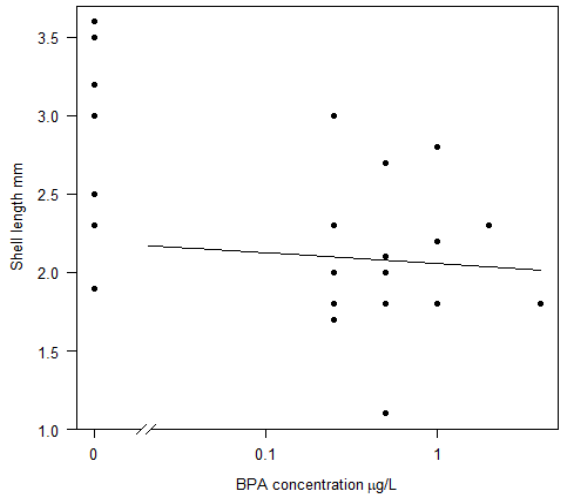



**Figure 4-10: Juvenile *M. tuberculata* shell length after exposure to Bisphenol A for 21 days.**

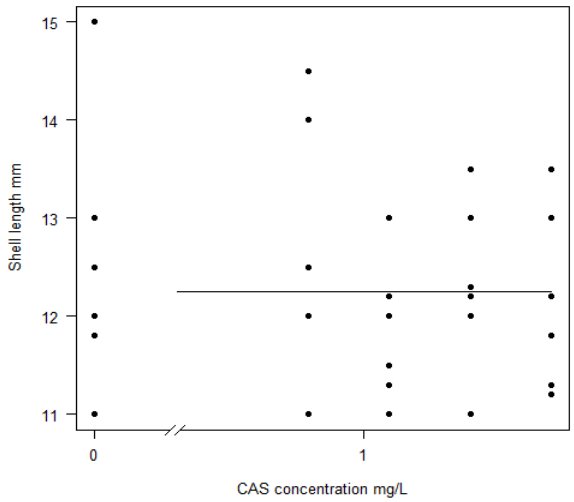



**Figure 4-11: Adult *M. tuberculata* shell length after exposure to calcium stearate for 21 days.**

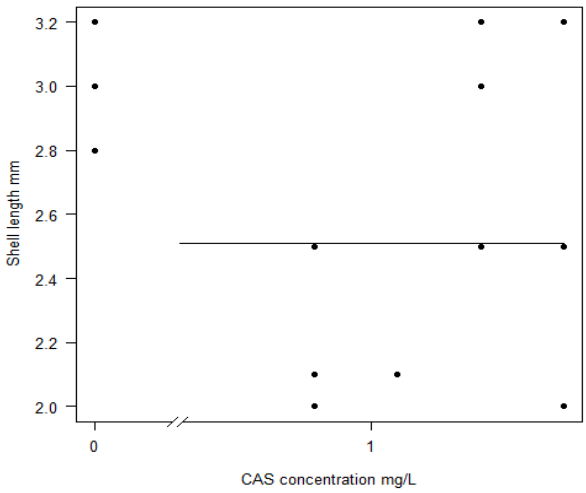



**Figure -12: Juvenile *M. tuberculata* shell length after exposure to calcium stearate for 21 days.**

Again, as in the other plasticisers assessed, the control shell length was greater than exposed shell lengths. However, variation in the data was high, and no clear pattern with increasing calcium stearate concentrations was apparent. Attempts to fit an exponential model rather than a log-logistic one were not able to converge on a solution.

### 4.3.5 Effect of different plasticisers on shrimp growth

Carapace lengths in *C. nilotica* exposed to varying concentrations of Bisphenol A for 21 days are presented in Figure 4-13. The curve fitting algorithm was not able to fit a statistically significant response to the plasticiser, largely owing to increased variation in the data, particularly at higher exposure concentrations. No clear growth response of *C. nilotica* juveniles exposed to calcium stearate was found (Figure 4-14). Variation in the data and the lack of any apparent trend meant that the curve fitting algorithm was unable to fit a response curve to the data. The shrimp cultures held at IWR collapsed, and no shrimp were available to assess the effect of dibutyl phthalate on shrimp growth.

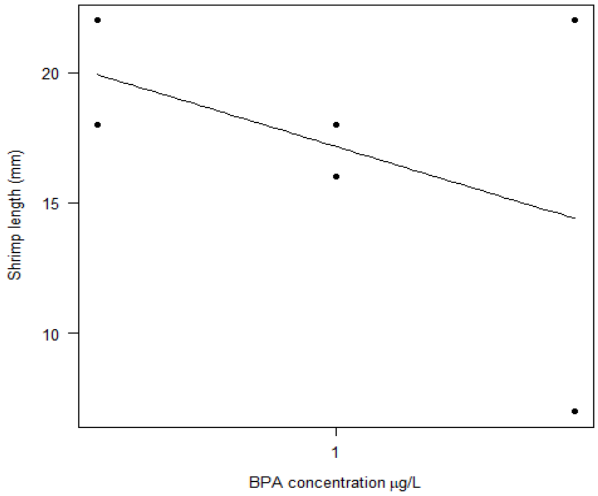



**Figure 4-13: Juvenile *C. nilotica* carapace length after exposure to Bisphenol A for 21 days.**

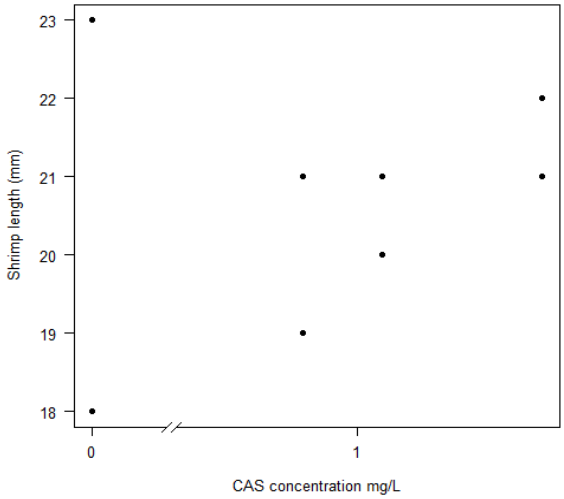



**Figure 4-14: Juvenile *C. nilotica* carapace length after exposure to calcium stearate for 21 days.**

### 4.3.6 Effect of different plasticisers on freshwater fish Tilapia

#### *4.3.6.1 Tilapia growth-length*

The response of juvenile *T. sparrmanii* body length to immersion in a Bisphenol A solution for 21 days is shown in Figure 4-15. Statistical methods were unable to fit a curve to the response depicted. Inspection of the data does not show a classic log-logistic response, and it appears that there was no growth response in *T. sparmanii* under test conditions. The response of juvenile *T. sparrmanii* to a range of concentrations of dibutyl phthalate is shown in Figure 4-16. Here again the curve fitting algorithms deployed could not generate a curve that shows a dose-response relationship. Inspection of the data presented in Figure 4-16 does not indicate any response of fish growth within 21 days to this plasticiser. Finally, the length of juvenile *T. sparrmanii* exposed to calcium stearate for 21 days is shown in Figure 4-17. As with the other plasticisers assessed, calcium stearate had no detectable influence on growth in this fish over 21 days, and inspection of the data shows no detectable response to this plasticiser.

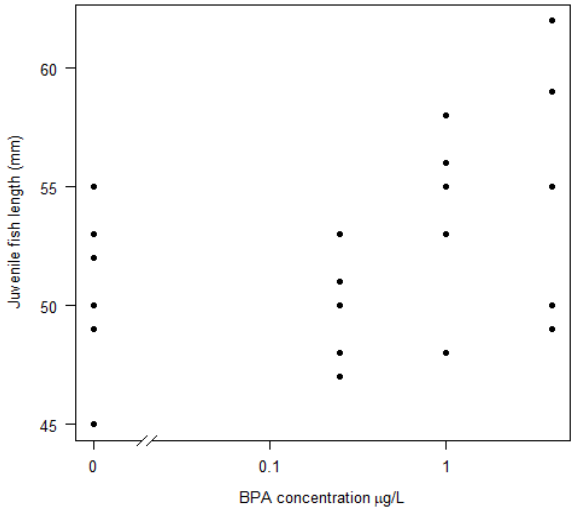



**Figure 4-15: Juvenile *T. sparrmanii* body length after exposure to Bisphenol A for 21 days.**

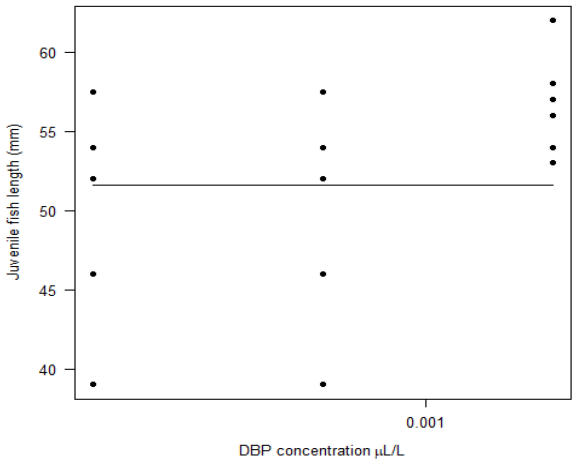



**Figure 4-16: Juvenile *T. sparrmanii* body length after exposure to dibutyl phthalate for 21 days.**

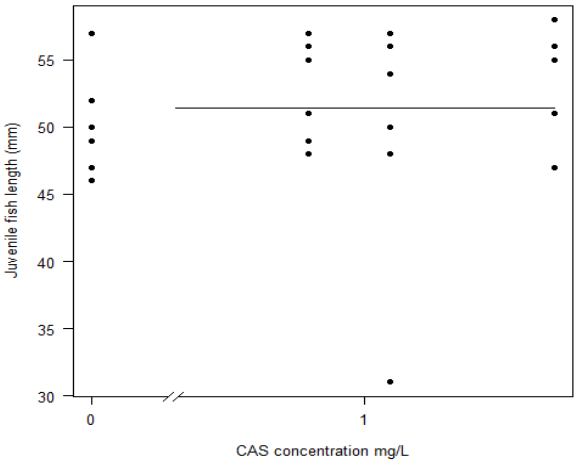



**Figure 4-17: Juvenile *T. sparrmanii* body length after exposure to calcium stearate for 21 days.**

#### *4.3.6.2 Tilapia growth-breadth*

The body width of juvenile *T. sparrmanii* after 21 days in a number of Bisphenol A solutions is presented in Figure 4-18. The fitted model indicates an increase in breadth with higher concentrations of Bisphenol A. However, as is apparent in Figure 4-18, the fitted model does not fit all the data, causing it to indicate an artificially high response. The model was not found to have a statistically significant fit to the data in this case. However, there seems to be a slight increase in breadth with increased plasticiser. Repeating the experiment with greater replication might reveal some growth response or establish whether this is an artefact.

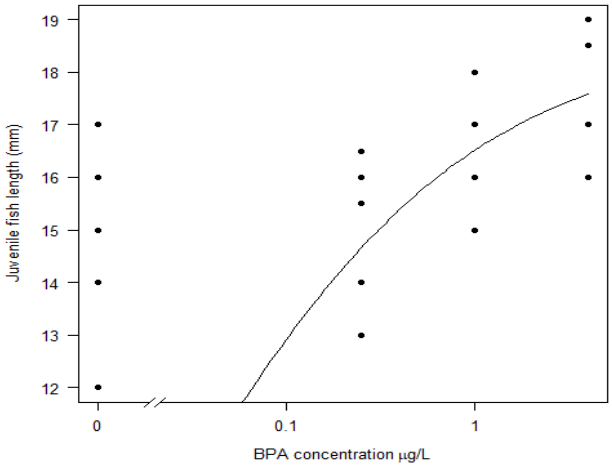



**Figure 4-18: Juvenile *T. sparrmanii* body breadth after exposure to Bisphenol A for 21 days.**

The response of juvenile *T. sparrmanii* width after 21 days of exposure to dibutyl phthalate is presented in Figure 4-19. Although the fitted model suggests that an increase with width occurred, there was no statistical support for this owing to the high variation between fish in the process. The response of juvenile *T. sparrmanii* width after 21 days of exposure to calcium stearate is presented in Figure 4-20. Although a model could be fitted, it indicated no change in width due to calcium stearate exposure, and

the fitted model could be distinguished statistically from a null model (p=0.434). Once again, variation in data overwhelmed any trends that might have been expressed.

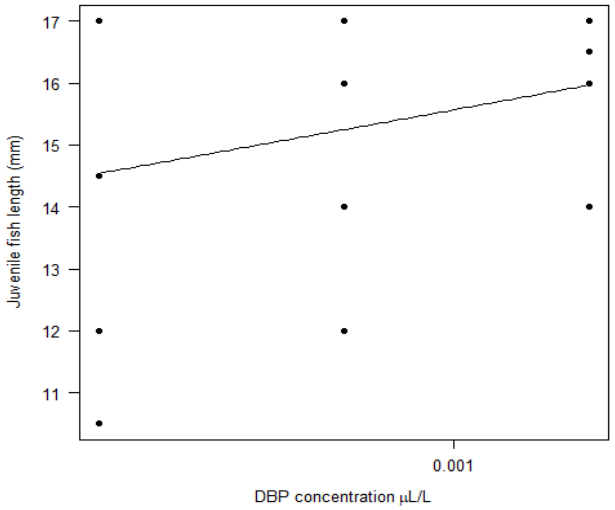



**Figure 4-19: Juvenile *T. sparrmanii* body breadth after exposure to dibutyl phthalate for 21 days.**

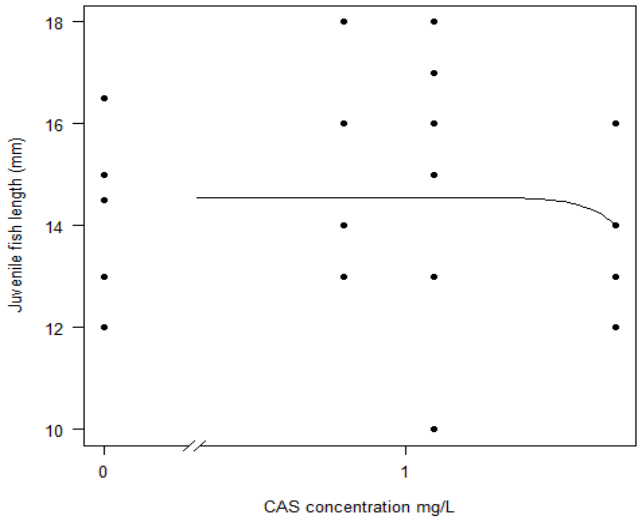



**Figure 4-20: Juvenile *T. sparrmanii* body breadth after exposure to calcium stearate for 21 days.**

### 4.3.7 Effects of plasticisers on enzyme activities in shrimp

#### *4.3.7.1 Acetylcholinesterase activity-shrimp*

The dose-response curve of acetylcholinesterase activity in juvenile *C. nilotica* exposed to calcium stearate for 21 days is shown in Figure 4-21. The fitted curve reflects an apparent log-logistic dose-response, and this curve is used to estimate the $EC_{50}$ and $EC_{10}$ concentrations. Inspection of Figure 4-21 reveals an anomaly, however. Compared to the EDTA control, there is no clear response to calcium stearate in the plotted data, with the sole exception of one data point at 5 mg/l, which shows a large

response. The other sample from the same concentration shows no clear response, which calls the validity of the apparent response and the $EC_{50}$ and $EC_{10}$ values into doubt. The goodness of fit test for this regression model shows it to be not a significant fit (p=0.646).

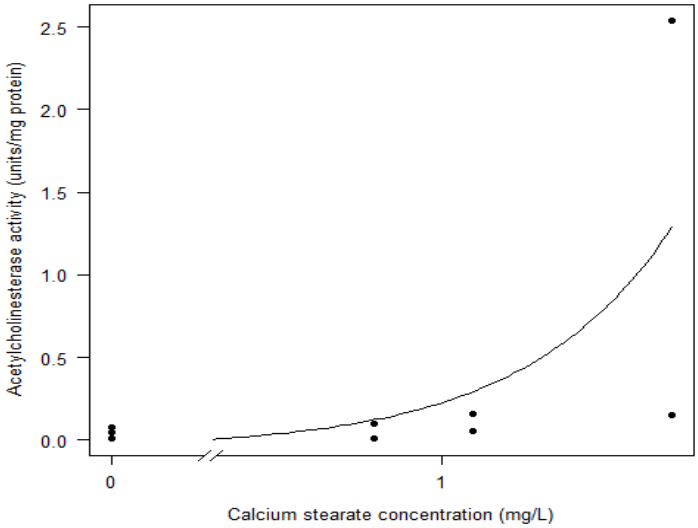



**Figure 4-21: Dose-response curve showing acetylcholinesterase activity in juvenile shrimp in varying concentrations of a calcium stearate suspension.**

The dose-response curve of acetylcholinesterase activity in juvenile *C. nilotica* exposed to Bisphenol A for 21 days is shown in Figure 4-22. The fitted curve, which was used to estimate $EC_x$ values, shows a roughly linear relation between log concentration and acetylcholinesterase activity. In Figure 4-22, it is clear that a greater response of acetylcholinesterase activity was found at 0.25 mg/l than at 1.0 mg/l, which adds to variation in the response. The overall acetylcholinesterase activity was high at 4 mg/l Bisphenol A, and it seems that this enzyme activity is stimulated overall by Bisphenol-A. The line fit was not statistically significant at 5%, but with p=0.126, it suggests that greater replication might return a statistically significant fit.

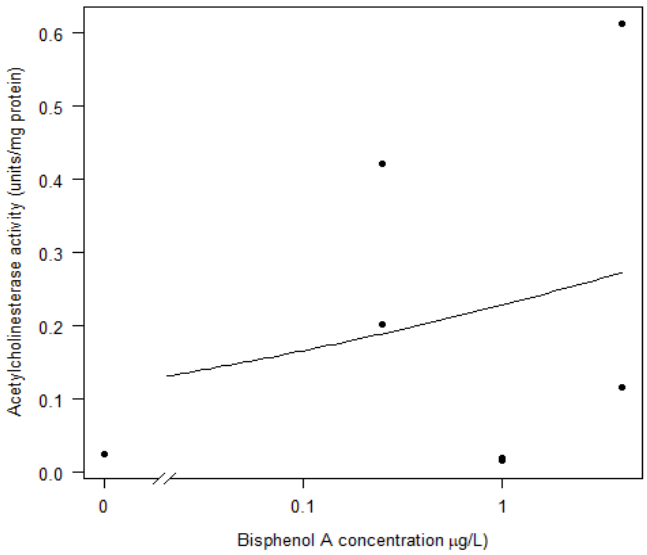



**Figure 4-22: Dose-response curve showing acetylcholinesterase activity in juvenile shrimp in varying concentrations of a Bisphenol A suspension.**

The dose-response curve of acetylcholinesterase activity in juvenile *C. nilotica* exposed to dibutyl phthalate for 21 days is shown in Figure 4-23. The data points show a slow increase in acetylcholinesterase activity with increasing dibutyl phthalate, with one data point at 2.152 nL/L dibutyl phthalate having notably more acetylcholinesterase activity than the other. The fitted curve, which is significantly influenced by the high 2.152 nL/L response, shows a roughly log-logistic response to dibutyl phthalate. This model, which was the best of several assessed and provides the estimates of $EC_x$ values, did not provide a good fit to the data (p=0.649).

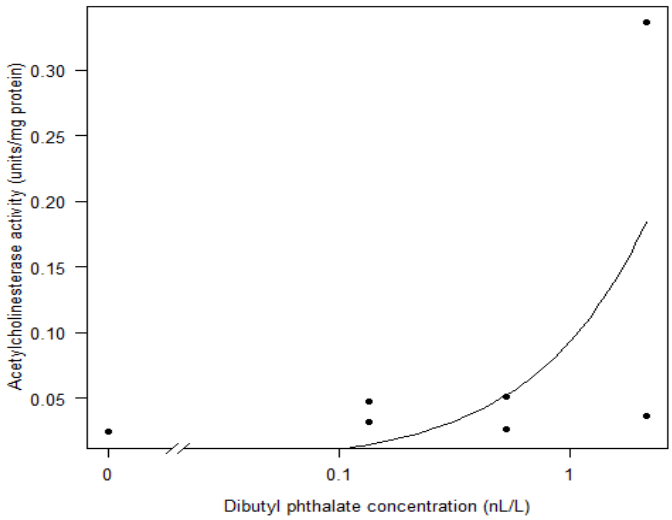



**Figure 4-23: Dose-response curve showing acetylcholinesterase activity in juvenile shrimp in varying concentrations of a dibutyl phthalate suspension.**

Differences between acetylcholinesterase activity per unit protein in shrimp between different plasticisers were not significant alone (p=0.461), but the effect of changing concentrations of the different plasticisers was statistically significant (p=0.007). Model significance supports the conclusion that acetylcholinesterase responded to the different plasticisers used (p=0.003). Similar results were found for lipid peroxidase. Alone, the different plasticisers could not be found to have a significant impact on the stress protein activity (p=0.517). Still, different concentrations of different plasticisers significantly impacted lipid peroxidase activity (p=0.011). The model significance supports the conclusion that plasticisers modify stress protein activity (p=0.007).

#### *4.3.7.2 Lipid peroxidase activity-shrimp*

The dose-response curve of lipid peroxidase activity in juvenile *C. nilotica* exposed to calcium stearate for 21 days is shown in Figure 4-24. The fitted curve shows an exponential increase in lipid peroxidase activity with increased exposure to calcium stearate. Compared to the EDTA controls, no significant change in lipid peroxidase activity occurred at 1.25 mg/l of calcium stearate, but after that activity increased. The fitted model that was used to generate $EC_x$ value estimates exhibited a statistically insignificant fit at p=0.709.

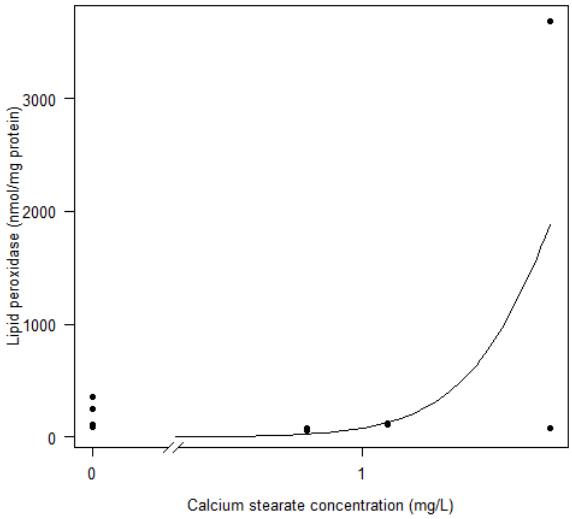



**Figure 4-24: Dose-response curve showing lipid peroxidase activity in juvenile shrimp in varying concentrations of a calcium stearate suspension.**

The dose-response curve of lipid peroxidase activity in juvenile *C. nilotica* exposed to Bisphenol A for 21 days is shown in Figure 4-25. The response presented here is unusual in that small doses of Bisphenol A seem to inhibit lipid peroxidase activity, and greater doses then lead to increase inactivity. The control showed lipid peroxidase activity at the same level as controls in other experiments. Therefore, this can be taken as a base level of lipid peroxidase in *C. nilotica* under experimental conditions. Therefore, the decrease in enzyme activity is to a level lower than the resting level, and as Bisphenol A increases, enzyme activity returns to normal. Of the ten toxicological models that were fitted, none was able to fit the patterns shown by the data. The curve shown in Figure 4-25 was a log-logistic model, with a not quite statistically significant fit to the data (p=0.095). The response of this enzyme system to Bisphenol A deserves further research.

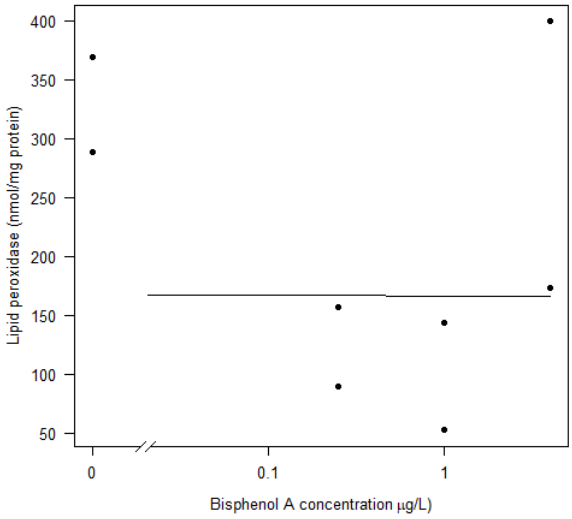



**Figure 4-25: Dose-response curve showing lipid peroxidase activity in juvenile shrimp in varying concentrations of a Bisphenol A suspension.**

The dose-response curve of lipid peroxidase activity in juvenile *C. nilotica* exposed to dibutyl phthalate for 21 days is shown in Figure 4-26. The figure shows a trend apparent in the responses of the enzyme systems assessed to increasing levels of plasticiser, in that lower levels of dibutyl phthalate had little effect on the enzyme system assessed, but at the higher levels, one of the two replicates had a strong response to the plasticiser, while the other replicate showed little or no response. As a result of the strong response, a shifted Michaelis-Menten dose-response which fitted best (p=0.475) was used to generate the $EC_x$ values. The data does not support the model, and predictions made using the model will be suspect.

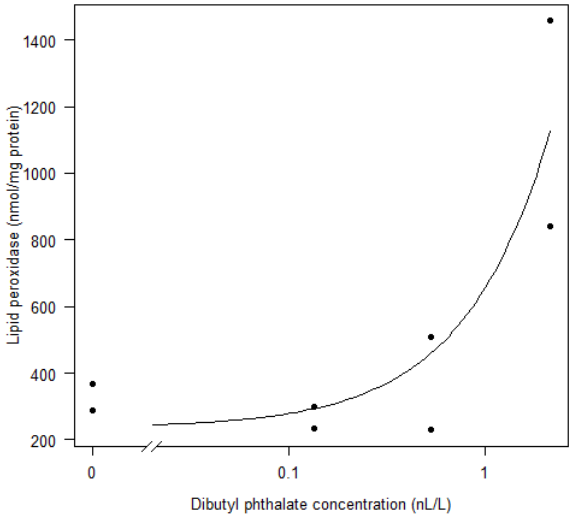



**Figure 4-26: Dose-response curve showing lipid peroxidase activity in juvenile shrimp in varying concentrations of a dibutyl phthalate suspension.**

### 4.3.8 Effects of plasticisers on enzyme activities in freshwater fish Tilapia

#### *4.3.8.1 Acetylcholinesterase activity-fish*

The dose response curve for acetylcholine esterase activity for *T. sparrmanii* exposed to calcium stearate for 21 days is shown in Figure 4-27. The figure shows a distinct acetylcholinesterase response to the plasticiser, with increasing levels of resulting levels of plasticiser resulting in increased acetylcholinesterase activity. The data have relatively low variation, which leads to a significant model fit to the data (p=0.005). The response of acetylcholinesterase to dibutyl phthalate exposure is presented in Figure 4-28. As in Figure 4-28, the response of acetylcholinesterase in fish in response to increasing levels of dibutyl phthalate is clear, and relatively little variation in the data is apparent. Despite this, the fit of the statistical model is not significant at the commonly accepted 5% level (p=0.092), probably as the control responses were not well modelled. The response of acetylcholinesterase activity in fish to increasing levels of Bisphenol A is shown in Figure 4-29. As in all the acetylcholinesterase responses in fish to plasticiser exposure, the response to increased plasticiser levels is clear and shows relatively little variation in comparison to many of the responses reported on here.

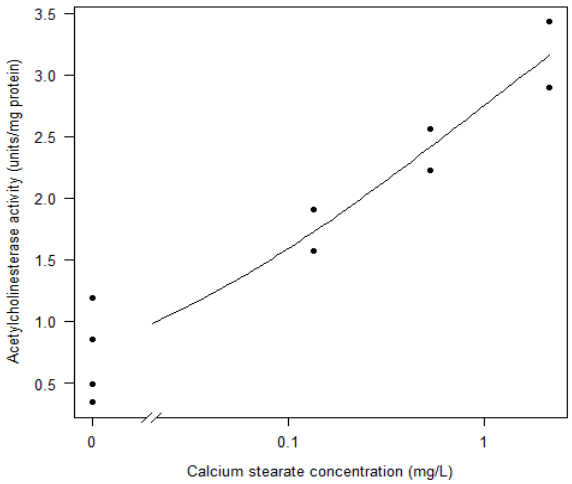



**Figure 4-27: Dose-response curve showing acetylcholinesterase activity in juvenile fish in varying concentrations of a calcium stearate solution.**

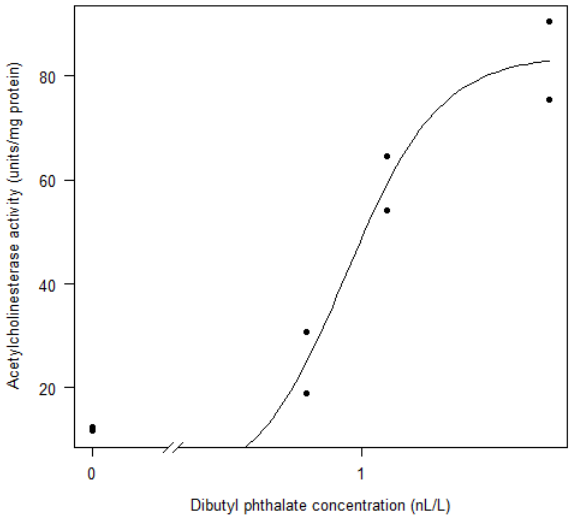



**Figure 4-28: Dose-response curve showing acetylcholinesterase activity in juvenile fish in varying concentrations of a dibutyl phthalate solution.**

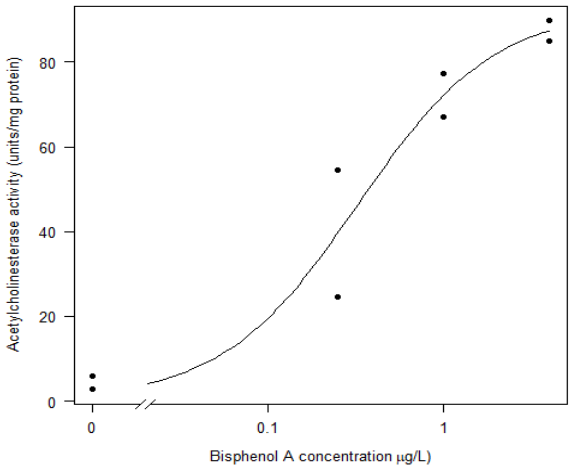



**Figure 4-29: Dose-response curve showing acetylcholinesterase activity in juvenile fish in varying concentrations of a Bisphenol A solution.**

In fish, statistically significant differences between plasticiser exposure in the expression of acetylcholinesterase were found. The enzyme system responded differently to the different plasticiser ($p<0.001$), and also to differing concentrations of the various plasticisers ($p<0.001$). Not surprisingly, strong statistical support for the derived model was found ($p<0.001$). Support for a response of lipid peroxidase to different plasticiser was also present ($p<0.001$), although no significant effect of plasticiser concentration were found ($p=0.325$). The overall model strongly supports a response of lipid peroxidase to plasticiser presence ($p<0.001$). Owing to a lack of material, no tests on snail enzyme systems could be undertaken.

#### *4.3.8.2 Lipid peroxidase-fish*

The response of lipid peroxidase in fish to exposure to calcium stearate is presented in Figure 4-30. Increased calcium stearate leads to a roughly log linear response, and levels of lipid peroxidase show a clear increase when exposed to increasing levels of this plasticiser. The model fit of this curve is clearly statistically significant ($p<0.001$). The response of lipid peroxidase in fish to increasing levels of dibutyl phthalate are presented in Figure 4-31. While an increase from control levels is present, the response is limited and seems not to respond to increases in the level of plasticisers present beyond showing an increase from control levels. As a result, the goodness of fit test of the fitted statistical model is not significant ($p=0.854$). The response of lipid peroxidase activity in fish to Bisphenol A exposure was not clear (Figure 4-32). Apart from very high variation at lower levels of the plasticiser, no clear response at higher levels was apparent. The trajectory of the curve suggest that higher levels of plasticiser might lead to a response in this enzyme system, but this would require further experimentation.

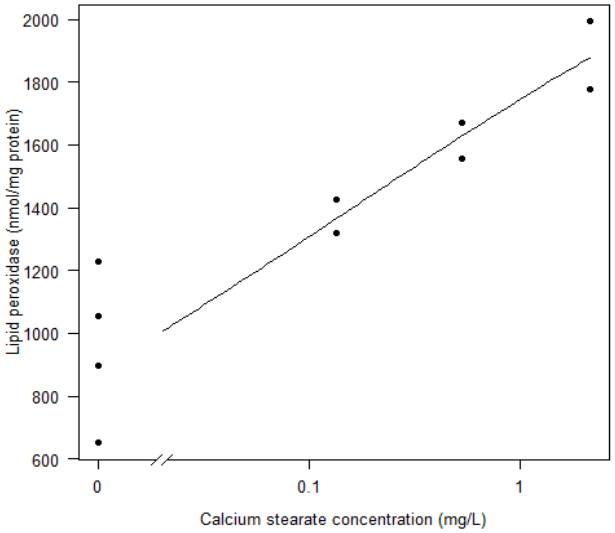



**Figure 4-30: Dose-response curve showing lipid peroxidase activity in juvenile fish in varying concentrations of a calcium stearate solution.**

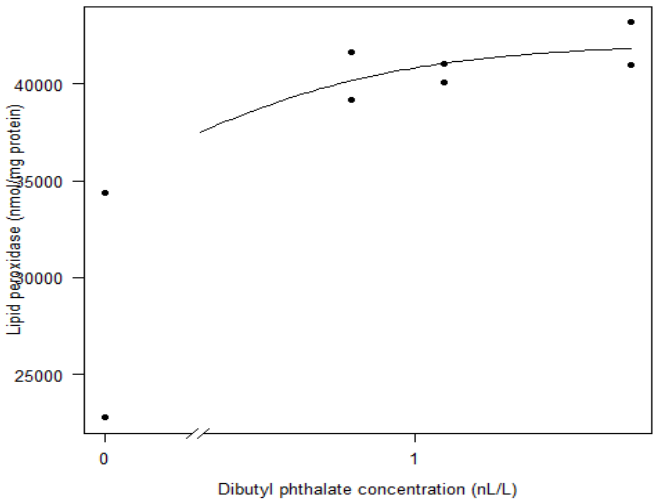



**Figure 4-31: Dose-response curve showing lipid peroxidase activity in juvenile fish in varying concentrations of a dibutyl phthalate solution.**

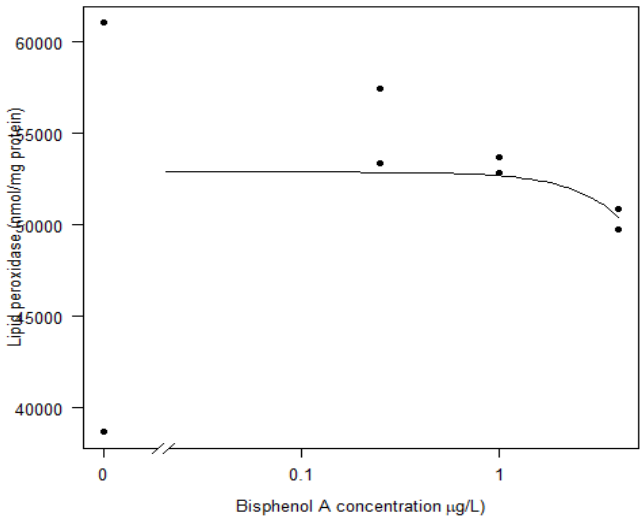



**Figure 4-32: Dose-response curve showing lipid peroxidase activity in juvenile fish in varying concentrations of a Bisphenol A solution.**

## 4.4 DISCUSSION

The major result from the tests presented here is the limited toxicity attributable to the plasticisers dibutyl phthalate, Bisphenol A and calcium stearate. The majority of the curves do not show any clear trend with increasing plasticiser levels. The most notable exception was the production of offspring by *M. tuberculata* when exposed to Bisphenol A, which showed a clear decrease in reproductive success with increased Bisphenol A. The other plasticisers had no clear effect on snail reproductive success. Comparisons between the various treatments were also not clearly statistically distinguishable, despite the different chemical nature of the plasticisers. This adds further support to a general observation that most of the plasticisers tested had a little detectable impact on reproduction and growth in the test taxa in the quantities tested. However, despite the impact of plasticisers being difficult to statistically separate from other background variations, in a few cases, plasticiser impacts were found. The clearest is the response of snail reproduction, which was severely inhibited after 21 days in the presence of Bisphenol

A. This result indicates that plastics with a Bisphenol A plasticiser could plausibly have a negative environmental impact by leaching the plasticiser.

Fish egg hatching was another endpoint that seemed to respond to plasticiser levels. However, the apparent response was not located within the range of tested concentrations. Both dibutyl phthalate and calcium stearate elicited a downturn in egg hatching at the highest plasticiser levels. The growth of juvenile *M. tuberculata* in varying plasticiser levels may have been modified by plasticiser levels, but the results were not clear, and fitting statistical models to the responses were often not successful. In all cases, the average shell length in snails from the control was higher than that from plasticiser treatments. However, there was no clear trend in shell length in response to increasing plasticiser concentrations. These results, as they stand, cannot be said to clearly indicate the sensitivity of *D. rerio* egg hatching to plasticiser concentrations. Still, they do indicate that further research will be of value in resolving this issue.

Other growth responses to the plasticisers tested revealed no clear growth response. Data returned were often very variable, which limited the effectiveness of curve fitting procedures to fit a curve and thereby return useful $EC_x$ values. Even where a clear dose-response curve was not returned from the test procedure, it is important to note that comparisons of test sensitivity, it was often possible to identify an impact of plasticiser tested alone or the interaction of plasticiser type and concentration. In some cases, a comparison of the generated statistical model with a null model did not reveal a significant difference, indicating that the models where significant effects of plasticiser type were returned, these models were not able to account for much of the variation found in the data. Greater replication may allow for clearer conclusions. However, while there are some detectable effects on plasticisers on different endpoints, the responses are not large, and in general, growth responses were not detected.

In terms of the analyses presented here, which assess the potential impacts of plasticisers on various facets related to reproduction and growth in fish, shrimp and snails, it appears that even where the effects of the plasticisers are not easily quantified or modelled, they are detectable and can be distinguished from each other. This raises the question of whether the levels of plasticiser used in tests are environmentally realistic. As noted in the methods, the levels of plasticiser assessed here are, at most, slightly higher than reported environmental levels where such data exist. In the most pronounced and clear response found here, the reproductive success of *M. tuberculata* after 21 days of exposure to Bisphenol A, the $EC_{50}$ was 0.61 µg/L, an amount that can easily be found in the environment (e.g. see Huang *et al*., 2012). The $EC_5$ from the results presented here is 0.06 µg/L, and this represents the concentration at which Bisphenol A will reduce *M. tuberculata* reproduction by 5%. According to Huang *et al*. (2012), this level is common in the environment, and as a result, the data presented here suggest that environmental impacts of Bisphenol on snail reproduction may be significant at current levels.

The data from the other tests are more ambiguous. The fish egg hatching test result suggests that fish reproduction might be hampered at approximately 0.001 µL/L of dibutyl phthalate or approximately 1.2 mg/L of calcium stearate. These are higher than environmental levels of dibutyl phthalate reported by Edjere *et al*. (2016), and comparative calcium stearate levels were difficult to locate; the impact of the tested levels based on the tests assessed here is not easy to predict. However, calcium stearate is used as a food additive, and toxicity is limited, while there are concerns about dibutyl phthalate in the environment. In conclusion, of the various tests assessed, growth could not be clearly modified by exposure to the plasticisers that were assessed. Reproduction in the test taxa might be more threatened by plasticiser exposure, particularly in the snail *M. tuberculata*.

Data presented here assesses the impact of selected plasticisers on two common stress enzyme systems. The first is acetylcholinesterase, which breaks down acetylcholine in neural synapses. The second is lipid peroxidase, which controls oxidative degradation of polyunsaturated lipids.

The results of the tests presented here reveal that, in general, plasticisers seem to impact on stress enzyme activity. Given the lack of significant response of most endpoints to the plasticisers in other tests and the near absence of clear responses to physical exposure to microplastics, it is of value to identify one pathway that may lead to microplastic impacts in the environment. Stress protein activity is a sub-lethal, chronic response to plasticiser exposure, and impacts on these enzyme systems indicate an increased probability of other detectable impacts on different endpoints. Changes in acetylcholinesterase activity will modify acetylcholine levels and consequently affect neural transmission and functioning, and changes to lipid peroxidation will modify the activity of this antioxidant. They may lead to oxidative damage to cell lipids, membranes, etc. Modifications to cell and neural activity at this level may affect any other endpoints, though predicting which one will not be easy. Nevertheless, an impact on basic regulatory enzymes such as acetylcholinesterase and lipid peroxidase is of considerable consequence for an organism exposed to these plasticisers.

Although detectable impacts of these plasticisers are noted here, there are problems with statistical support for the models and consequent $EC_x$ values presented here. Most notable, although the models fitted to the data indicate increased enzyme activity with increased plasticiser, in many cases, the response only occurred in one of two replicates and at the highest levels assessed. The end result of this was a lack of statistical support for some models and high standard errors on $EC_x$ values. This trend was common enough to result in multiple reassessments of the data, but only a few of these could detect any systematic error in the data, and it appears that the results are valid. However, although it seems that response occurred, the response of taxa to plasticisers needs further assessment with greater replication and more concentrations assessed to determine how plasticisers impact shrimp and other taxa.

Another consideration is that, although apparent responses were detected, in most cases, the effects were found at levels of plasticiser that would not easily be encountered in the environment. However, the levels that were tested were based on reported environmental levels.

Background information on calcium stearate was not easily found. This plasticiser has many uses, including many other uses, including as an ingredient in food and pharmaceutical products, a lubricant, use in waterproofing, and production of pulp and paper (Budavari, 1996; Ley, 2001; NCBI, 2019 and refs therein). The EPA classifies calcium stearate as adhesives and sealant chemicals, anti-adhesive agents, fillers, finishing agents, flame retardants, hydrophobic agents, intermediates, and lubricants lubricant additives, neutralizing agents, polymer stabilizer, processing aids, and surface-active agents (NCBI, 2019). Bayo *et al*. (2019, 2021) report on calcium stearate pellets in wastewater treatment works effluent, but these are not quantified, and the amount of dissolved calcium stearate is unknown. Although the use of calcium stearate in food suggests limited or no toxicity, the results presented here suggest that there may be an effect. However, an apparent response was present in one replicate only and then at the highest levels only, and nothing conclusive can be drawn from the results presented here. Bisphenol A levels have been reported from the environment at levels around the higher levels assessed here and at higher levels in industrial effluents and recycling leachate in China, Japan, Germany and Canada (Huang *et al*., 2012 and references therein). Significant levels of other Bisphenol analogues have also been reported (Chen *et al*., 2016 and references therein). This indicates that the responses to Bisphenol A presented in this report are likely in polluted freshwater sites, though not at other locations. If the Bisphenol A is derived from microplastics, the response would depend on the plasticiser's high enough leach rate from the microplastic.

Environmental levels of dibutyl phthalate have been assessed around the world. In many cases, levels found were lower than the highest levels assessed here (Gao and Wen, 2016; Oehlmann *et al*., 2008; Peinenburg and Struijs, 2006), but in some cases, environmental dibutyl phthalate levels exceeded the levels assessed in this research (Fatoki *et al*., 2010; Fatoki and Vernon, 1990; Fatoki and Noma, 2001). These references report on dibutyl phthalate levels in freshwater in many countries, including India, Spain, France, Korea, China, Canada, Netherlands, United Kingdom, and South Africa. Of these, high levels of dibutyl phthalate were most commonly reported from South Africa (Fatoki *et al*., 2010; Fatoki and Noma, 2001; Olujimi *et al*., 2010). Higher levels of dibutyl phthalate were commonly associated with discharge from wastewater treatment works (Fatoki and Noma, 2001; Olujimi *et al*., 2012). Although the dose-response curve fitting procedures' results were not highly significant, the apparent response, particularly of lipid peroxidase, to dibutyl phthalate is of concern given the high levels reported from South African rivers. Bisphenol A has been found to have endocrine effects (Allard, 2014; Miyagawa *et al*., 2016), and dibutyl phthalate may have endocrine activity (CDC, 2009). The tests undertaken here assessed the effect of the plasticisers on enzyme systems that control acetylcholine management and lipid oxidation and so do not reflect directly on endocrine action. However, it may contribute to changes in the enzyme systems assessed.

## 4.5 SUMMARY

Plasticisers are included in plastics in varying quantities (Cadogan and Howick, 2000; Godwin, 2011; Wypynch, 2017). Plasticisers can leach out of plastics, making the plastic more brittle and leading to plasticiser accumulation in the surrounding environment (e.g. Kastner *et al*., 2012; Erythropel *et al*., 2014; Jacobson *et al*., 1977; Zhang and Chen; 2014; Fromme *et al*., 2002; Vandenberg *et al*., 2007). However, the rate of plasticiser leaching will depend on the polymer mix with its additives, the age of the plastic particle, and other factors. For this reason, it is not straightforward to quantify the rate of plasticiser leaching from microplastics. The difference between the microplastic test results and the plasticiser test results indicates that plasticiser leaching did not make up a large part of microplastic toxicity. However, the leaching rate is speculative. In addition, an organism like a single-celled taxon that might attach to a microplastic particle is likely to be exposed to more plasticiser than another organism co-suspended in the same medium as the microplastic or plastic surface.

# CHAPTER 5: ASSESSING MICROPLASTICS OCCURRENCE AND DISTRIBUTION IN THE SWARTKOPS AND BUFFALO RIVER SYSTEMS

## 5.1 INTRODUCTION

The global annual production of plastics has increased rapidly in recent decades. For example, approximately 359 million tons were produced in 2018 (de Carvalho *et al*., 2021). Plastic production is expected to increase exponentially to compensate for the global population growth that is projected to be to be more than 9 billion by 2050 (United, World, and Development 2020; Yin *et al*., 2021)). Although plastic use is essential in many aspects of people's daily lives because of its convenience, most of the produced plastics enter the environment, causing various levels of the environmental population (Bertoli *et al*., 2022; Zhang *et al*., 2022). Inappropriate disposal and failing management policies to control plastic are the main reasons for plastic pollution (Angnunavuri *et al*., 2020; Ryan 2020). In particular, the aquatic environment receives most of the plastic materials via rainfall-runoff, sewage discharge, and atmospheric precipitation and produces several environmental problems in 2021 (Lin *et al*., 2021).

Environmental factors cause plastic materials to break into tiny particles called microplastics (5 mm in diameter) (Lin *et al*., 2021). As an emerging contaminant, microplastics while in the environment have become a significant concern in recent years (Fan *et al*., 2021), causing various environmental hazards in the marine (Ryan 2020; Ryan *et al*., 2020) atmosphere (Abduro Ogo *et al*., 2022; Yin *et al*., 2021), soil and freshwater (Bertoli *et al*., 2022) environments, and even in digestive organs of organisms (Bertoli *et al*., 2022; Pastorino *et al*., 2021). For example, microplastics ingested by organisms have been shown to block the digestive tract and cause physiological and morphological stress on organisms (Ferreira *et al*., 2016; Lusher *et al*., 2013; Van Cauwenberghe *et al*., 2015). Additionally, studies have found microplastics to be carriers of toxic contaminants due to their large surface areas to volume ratio and strong adsorption capacities (Tan *et al*., 2019). These toxic substances, such as EDCs, and heavy metals attached to microplastics, also pose significant hazards to aquatic organisms, affecting humans via biomagnification along the food chain (Zhang *et al*., 2019; Sanatana *et al*., 2017).

The distribution, occurrence and effects of microplastics in freshwater ecosystems have received considerable attention in other world regions (Murphy and Quinn 2018; Wang *et al*., 2021; Windsor *et al*., 2018). In South Africa, much of the research efforts on microplastic pollution have focused on marine ecosystems (e.g. Ryan, 2020). Microplastic abundances reported recently showed that microplastic pollution in some freshwater environments of South Africa was severe (Bulannga and Schmidt, 2022; Nel, Dalu, and Wasserman, 2018), especially in areas where anthropogenic activities were frequent. However, we still lack enormous monitoring data on the distribution and occurrence of microplastics in heavily urbanised rivers in the country.

The Swartkops and Buffalo Rivers in the Eastern Cape drain heavily urbanized landscapes (Odume, 2020). They are impacted by numerous anthropogenic activities, including effluents from municipal wastewater treatment works, run-off from informal settlements, industrial facilities and agricultural farmlands, and sites refuse disposals. As a result, elevated concentrations of nutrients, suspended and dissolved solids and depletion in dissolved oxygen have been consistently reported in these two catchments (Odume and Mgaba, 2016; Zuma, 2010). However, the distribution, occurrence, and impact of microplastics on river biodiversity have not been investigated. Therefore, this chapter explores the

distribution and abundance of microplastics in the Swartkops and Buffalo river systems, Eastern Cape Province of South Africa.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Study area and sampling sites

The study was conducted in two different rivers, the Buffalo River and the Swartkops River in the Eastern Cape (Figure 5-1). The Swartkops River flows through an urban catchment within the industrial town of Uitenhage and the residential town of Despatch in the Eastern Cape Province of South Africa. The river is impacted by both point and diffuse pollution sources, including effluent discharges from municipal wastewater treatment work, run-off from in-formal settlements, agricultural farmlands, and run-off from road and rail networks. The Buffalo River flows through these urban and suburban areas. The anthropogenic activities in these locations predispose factors to the discharge of agricultural, domestic, and industrial wastes (Yahaya *et al*., 2019). The Buffalo River covers about 1287 km$^2$ (Ohoro *et al*., 2021). The river receives hazardous wastes from an old tannery textile mill and leached toxic wastes from the dumpsite close to Zwelitsha (Yahaya *et al*., 2019). The river flows from Amathole Mountain and ends in the Indian Ocean via East London Creek. The sampling points in the locations were reported by Yahaya *et al*. (2017). Five sampling sites were selected on the Buffalo River (Site 1 or Eluphondweni, Site 2 or River Road, Site 3 or Zwelitsha, Site 4 or Potsdam, and Site 5 or Buffalo Pass). Seven sites were selected along the Swartkops River and named Sites 2 to 7. Site locations are in Figure 5-1.

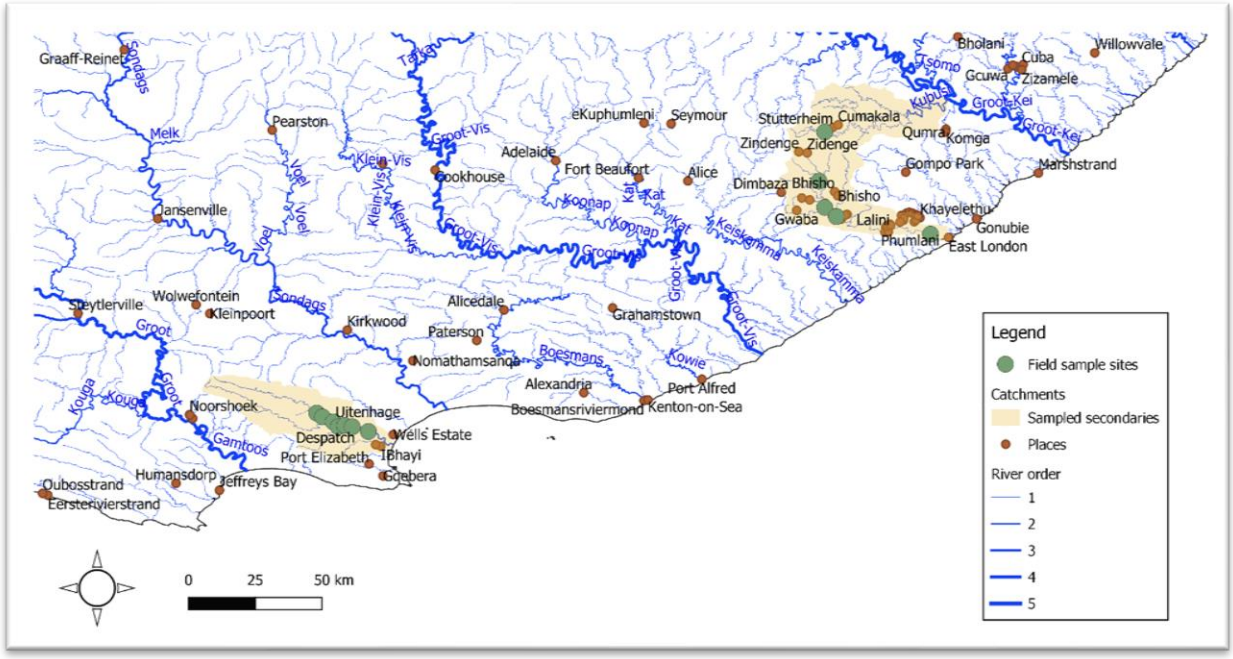



**Figure 5-1: Map showing field sampling sites in the Swartkops and Buffalo River catchments in the Eastern Cape.**

### 5.2.2 Water sampling and physiochemical analysis

For one hydrological year, four field trips were undertaken to the Buffalo River and Swartkops River sites, from July 2020 to June 2021. Electrical conductivity (EC), dissolved oxygen (DO), pH, and temperature were measured on-site using Hanna multiprobe meter for each trip to all sites. Water samples were collected using acid-washed glass bottles facing upstream to avoid sediments entering the sample. For each site, two samples were collected, one from pooled water and a second one from faster-moving water. Samples were then transported to the Institute for Water Research (IWR) water quality laboratory at Rhodes University and preserved in a refrigerator at a temperature of 4°C for analysis (UNEP/WHO, 1996).

### 5.2.3 Microplastics analysis and quantification

Water samples were analysed for microplastic characterisation following three steps, including digestion of organic material using potassium hydroxide (KOH) and hydrogen peroxide ($H_2O_2$), visual examination and counting using a compound microscope, and polymer identification using attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR).

#### *5.2.3.1 Wet sieving*

Water samples were first homogenised at 40˚C for 30 minutes. Two sieves with mesh pore sizes of 2000 µm and 63 µm were used to fractionate the microplastic particles in the environmental water samples. The sieves were stacked with the 2000 µm mesh on top and the 63 µm sieve below. As a result, targeted microplastic particles ranged from 63 µm to 2000 µm. Collected microplastic particles were then washed from the 63 µm sieve to a 500 ml glass beaker using 100 ml or less of ultrapure water filtered using a 0.2 µm glass fibre filter. This filtrate, which contained microplastic and other particles from 1l samples, was stored in glass with aluminium foil caps until digestion.

#### *5.2.3.2 Digestion methodology*

The digestion method was adapted from Campanale *et al*. (2020). A 10% potassium hydroxide solution was added to the sample in the glass beaker in the ratio of 1 part solution to 3 parts sample. The mixture was then heated on a magnetic stirrer heater at 48˚C for 24 hours. At the end of this, an equal volume of 15% hydrogen peroxide was added to the beaker, and the mixture was left at room temperature for 15 minutes. The supernatant was then vacuum filtered onto a 0.2 µm glass microfiber filter. The filtrate was then allowed to air dry while loosely covered with aluminium foil. The dried filters were examined for microplastic particles using a compound microscope to count particles and a scanning electron microscope to assess microparticle morphologies. The polymer types were identified using Fourier transform infrared spectroscopy.

#### *5.2.3.3 Visual microscopic examination and counting*

The filter paper was divided into four quadrants, and each quadrant was examined for microplastics using a positive control as the standard measure that contained polypropylene, polyethylene and polyvinyl chloride. The total number of microplastic particles on a given filter paper was obtained by adding the total obtained in each quadrant. The known sample volume allowed the calculation of microplastic particle concentrations.

#### *5.2.3.4 Polymer identification*

The polymer characterisation was done using a universal ATR-FTIR. The filters were examined directly with a binning factor of 4 and spectra resolution of 8 $cm^{-1}$.

### 5.2.4 Data analysis

Comparisons of environmental microplastic used a simple analysis of variance to test changes in environmental microplastics with location, date, and flow rate. Where responses were statistically supported, a Tukey HSD postdoc test was used to determine distinct levels. All statistics and plotting used R 4.0.3 (R core team 2020), together with the libraries drc (Ritz *et al*., 2015), car (Fox and Weisberg, 2019), lmtest (Zeileis and Hothorn, 2002), emmeans (Lenth, 2020) and plyr (Wickham, 2011).

## 5.3 RESULTS

### 5.3.1 Variation in microplastic levels at the different sites

Environmental microplastic data were collected from sites in the Swartkops and Buffalo Rover catchments in the Eastern Cape. After microplastic levels in collected water had been determined, the data were analyzed to determine whether trends were present in the data. A simple analysis of variance was not able to find statistically significant patterns in the quantity of microplastics collected by the river (p=0.674), by the site (p=0.075) or by flow rate (p=0.125). It should be noted that although there was no statistically significant difference between sites at the widely used p=0.050 limit, the collected data had a result that was close to this. Greater replication would likely reveal differences between sites. A box and whisker plot of data from the two catchments is presented in Figure 5-2. There is no appreciable difference between the two catchments, as the plot shows. Box and whisker plots of microplastic levels at sample sites in the Swartkops and Buffalo River catchments are shown in Figure 5-3.

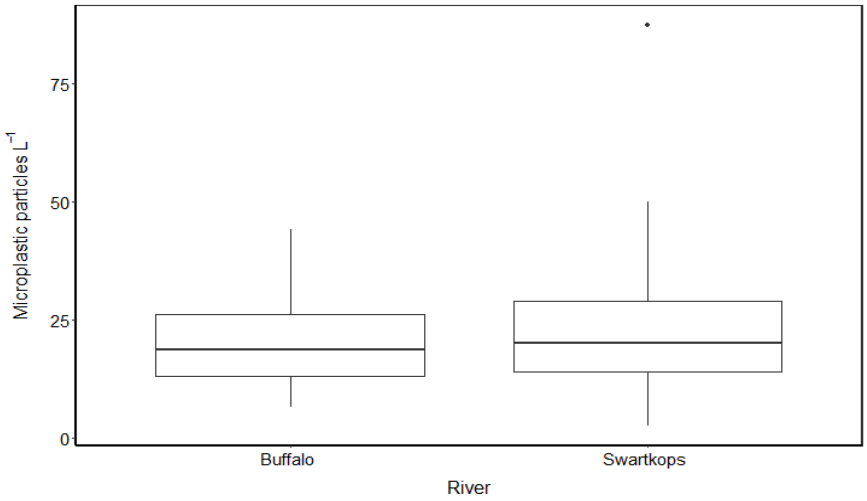



**Figure 5-2: Box and whisker plot of the microplastic data from the two catchments sampled.**

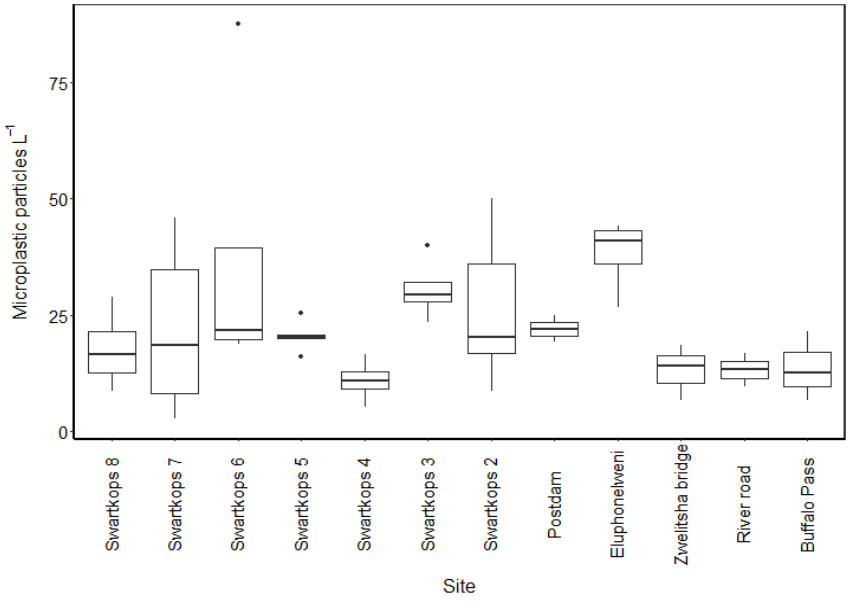



**Figure 5-3: Box and whisker plot of microplastic concentrations collected from the various sites sampled. Sites are listed from the top of each catchment to the bottom.**

For ease of interpretation, the sites are plotted from those higher in the catchments to those lower in the respective catchment. This plotting order was used in order to determine whether a trend might be present from less impacted upper catchment sites to lower catchment sites where levels of microplastic have increased owing to accumulative impacts. Inspection of Figure 5-3 reveals that no such trend exists and that upstream sites can have relatively high microplastic levels, while downstream sites can have low levels of microplastics.

### 5.3.2 Variation in microplastic levels under different flow conditions

Microplastic levels under differing flow conditions in the Swartkops and Buffalo Rivers are presented in Figure 5-4. As can be seen, the results from differing flow conditions cannot be clearly differentiated. This assessed whether well-mixed solutions might have more microplastics, while stiller water allowed for microplastic separation, either by sedimentation or by floatation, depending on the polymer density.

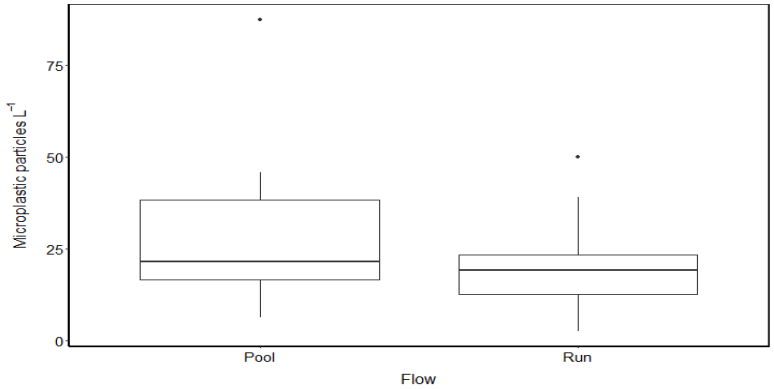



**Figure 5-4: Box and whisker plot of microplastic levels in differing flow conditions. Pools have low flow, while runs have distinct flow.**

### 5.3.3 Distribution of different microplastics across the sampled sites and hydraulic habitats

The distribution of different plastics across the sampled sites and hydraulic habitats is presented below in Figure 5-5 to Figure 5-9 below. When assessed on a per site per hydraulic habitat basis, the count of data is relatively low. From the plots, polypropylene particles were the most commonly encountered microplastic, polyvinyl chloride and polystyrene particles were the least frequent microplastic. In many cases the pool and run hydraulic habitats could not easily be distinguished in terms of the microplastic particles present; however, polyethylene tetraphthalate and polystyrene particles were more common in samples from pools then from runs.

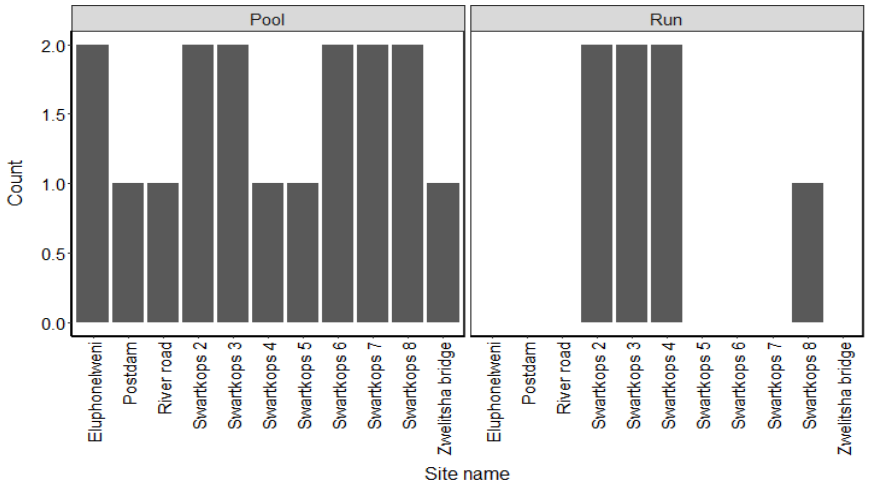



**Figure 5-5: Frequency of occurrence of polyethylene terephthalate particles in samples from all sample sites and hydraulic habitats.**

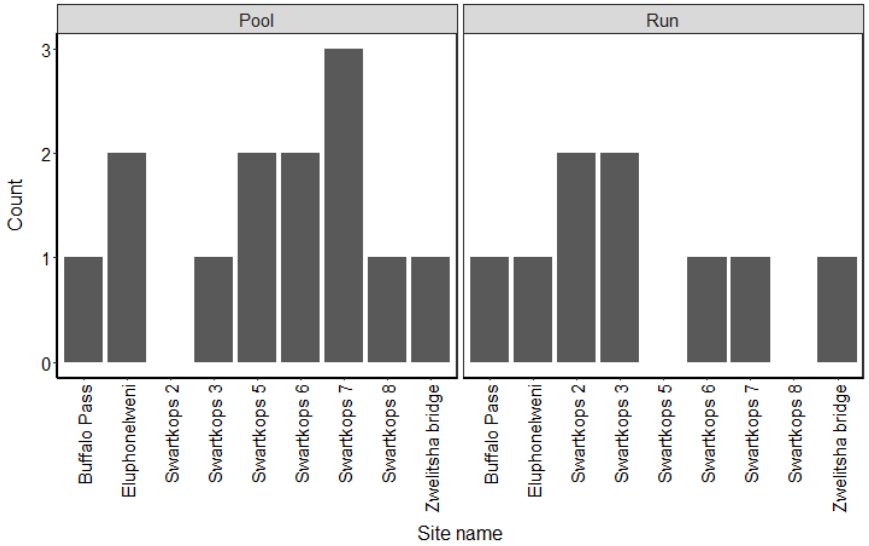



**Figure 5-6: Frequency of occurrence of polyethylene particles in samples from all sample sites and hydraulic habitats.**

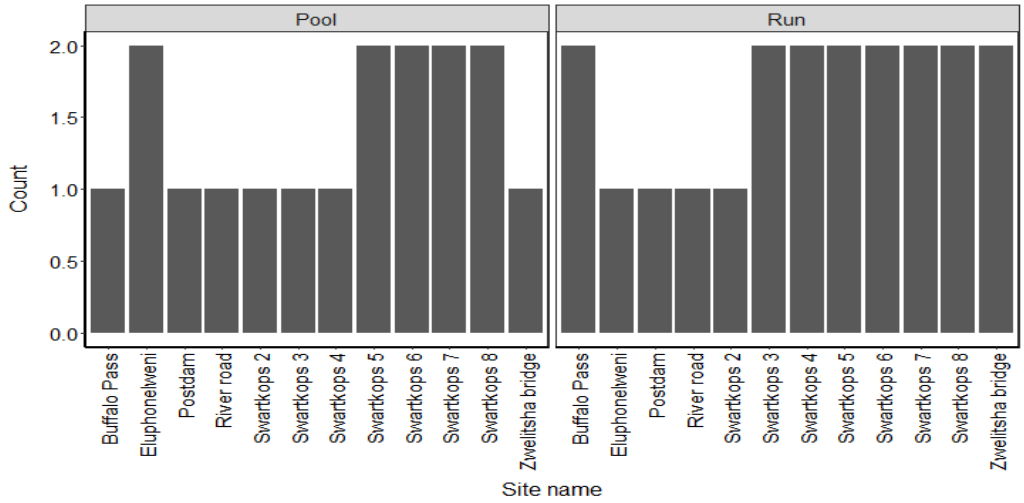



**Figure 5-7: Frequency of occurrence of polypropylene particles in samples from all sample sites and hydraulic habitats.**

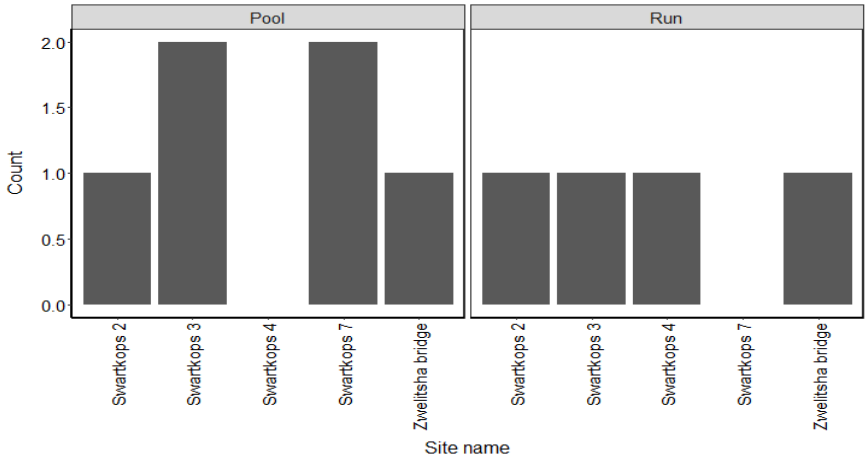



**Figure 5-8: Frequency of occurrence of polyvinyl chloride particles in samples from all sample sites and hydraulic habitats.**

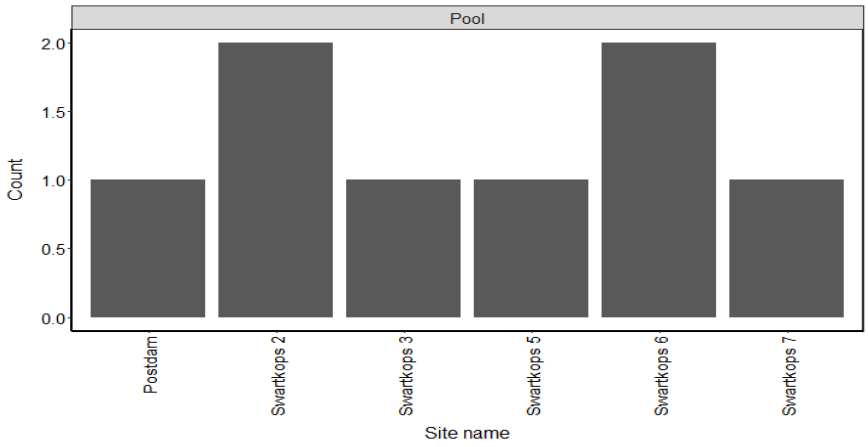



**Figure 5-9: Frequency of occurrence of polystyrene particles in samples from all sample sites and hydraulic habitats.**

## 5.4 DISCUSSION AND CONCLUSION

Microplastics were found in all samples reported here, illustrating their ubiquity in the sampled rivers. The plastic in microplastics was commonly evenly distributed across sites and hydraulic habitats, although some plastics were more common in pools rather than runs. The concentrations of microplastics found were fairly high compared to other sites in South Africa (Bouwman *et al*., 2018), or elsewhere in the world (Li *et al*., 2018; Luo *et al*., 2019; Stanton *et al*., 2020). High microplastic levels accord with the recent observation that plastic consumption is high and plastic waste management in South Africa fails (Zadan and de Kock, 2020). This observation raises the question of what such high levels of microplastics might mean for river biota. In the microplastic toxicology exposures undertaken in this project, these field concentrations were relatively low, and in laboratory exposures, no toxic impact was detected on a range of endpoints. These results accord with international observations that microplastic exposures that elicit a biotic response are often at levels of microplastics far higher than are encountered in the environment (Triebskorn *et al*., 2019; GESAMP, 2016; Tang, 2017; Lenz *et al*., 2016). In a similar light, European scientific advisers concluded that no known risks were posed by environmental levels of microplastics (SAPEA, 2019). It is, therefore, possible that the physical effect of this level of microplastic particles on biota in the environment may be limited or undetectable. However, plasticiser impacts do occur, and it would not be wise to write off environmental microplastic levels that are high on a global basis as being safe.

Overall, the environmental microplastic levels found were relatively high compared to data from South Africa and elsewhere. Nevertheless, when levels of environmental microplastics encountered are viewed in the light of toxicological test results, it seems unlikely that these will elicit a toxicological response in taxa like the ones assessed here. It remains possible that other taxa might have a negative response, a possibility perhaps more likely in an organism that has a feeding or behavioural strategy that maximizes its exposure to environmental microplastics. Nevertheless, the data presented here do not indicate a response to microplastics.

# CHAPTER 6: RESULTS SYNTHESIS, CONCLUSION AND RECOMMENDATIONS

## 6.1 DO MICROPLASTICS POSE A SIGNIFICANT RISK TO FRESHWATER ORGANISMS?

The results of the tests presented here reveal that, in general, plasticisers seem to impact on stress enzyme activity, though many other endpoints showed little response. Given the lack of significant response of most endpoints to the plasticisers in other tests, and the near absence of clear responses to physical exposure to microplastics, it is of value to identify one pathway that may lead to microplastic impacts in the environment. Stress protein activity is a sub-lethal, chronic response to plasticiser exposure, and impacts on these enzyme systems indicate and increased probability of other detectable impacts on other endpoints. Changes in acetylcholinesterase activity will modify acetylcholine levels and consequently have an effect on neural transmission and functioning, and changes to lipid peroxidation will modify activity of this antioxidant, and may lead to oxidative damage to cell lipids, membranes, etc. Modifications to cell and neural activity at this level may affect any number of other endpoints, though predicting which one will not be easy. Nevertheless, an impact on basic regulatory enzymes such as acetylcholinesterase and lipid peroxidase are of considerable consequence for an organism exposed to these plasticisers.

Although detectable impacts of these plasticisers are noted here, there are problems with statistical support for the models and consequent ECx values presented here. Most notable, although the models fitted to the data indicate increased enzyme activity with increased plasticiser, in many cases the response only occurred in one of two replicates and at the highest concentrations assessed. The end result of this was a lack of statistical support for models, and high standard errors on ECx values. This trend was common enough to result in multiple reassessments of the data, but none of these were able to detect any systematic error in the data, and it appears that the results are valid. However, although it seems that a response occurred, the response of taxa to plasticisers needs further assessment with greater replication and/or more concentrations assessed to determine how plasticisers impact on shrimp and other taxa.

Another consideration is that, although apparent responses were detected, in most cases the effects were found at levels of plasticiser that would not easily be encountered in the environment. However, the concentrations that were tested were based on reported environmental levels. Background information on calcium stearate was not easily found. This plasticiser has many uses, including as an ingredient in food and pharmaceutical products, as well as a lubricant, use in waterproofing, and production of pulp and paper (Budavari, 1996; Ley, 2001; NCBI, 2019d). The EPA classifies calcium stearate as being used as adhesives and sealant chemicals, anti-adhesive agents, fillers, finishing agents, flame retardants, hydrophobic agent, intermediates, lubricants and lubricant additives, neutralizing agent, polymer stabilizer, processing aids, and surface-active agents (NCBI, 2019e). Bayo *et al*. (2019, 2021) report on calcium stearate pellets in wastewater treatment works effluent, but these are not quantified, and the amount of dissolved calcium stearate is unknown. Although the use of calcium stearate in food suggests limited or no toxicity, the results presented here suggest that there may be an effect. However, an apparent response was present in one replicate only, and then at highest levels only, and nothing conclusive can be drawn from the results presented here.

Bisphenol A levels have been reported from the environment at levels around the higher levels assessed here, and at higher levels in industrial effluents and recycling leachate in China, Japan, Germany and Canada (Huang *et al*., 2012 and references therein). Significant levels of other bisphenol analogues have also been reported (Chen *et al*., 2016 and references therein). This indicates that the responses to bisphenol A presented in this report are likely in polluted freshwater sites, though not at other locations. If the bisphenol A is derived from microplastics, the response would depend on a high enough leach rate of the plasticiser from the microplastic.

Environmental levels of dibutyl phalate have been assessed around the world. In many cases, levels found were lower than the highest levels assessed here (Gao and Wen, 2016; Oehlmann *et al*., 2008; Peinenburg and Struijs, 2006), but in some cases environmental dibutyl phthalate levels exceeded the levels assessed in this research (Fatoki *et al*., 2010; Fatoki and Vernon, 1990; Fatoki and Noma, 2001). These references report on dibutyl phthalate levels in freshwater in many countries, including India, Spain, France, Korea, China, Canada, Netherlands, United Kingdom, and South Africa. Of these, high levels of dibutyl phthalate were most commonly reported from South Africa (Fatoki *et al*., 2010; Fatoki and Noma, 2001; Olujimi *et al*., 2010). Higher levels of dibutyl phthalate were commonly associated with discharge from wastewater treatment works (Fatoki and Noma, 2001; Olujimi *et al*., 2012). Although the results from the dose-response curve fitting procedures was not highly significant, the apparent response, particularly of lipid peroxidase, to dibutyl phthalate is of concern given the high levels reported from South African rivers.

Bisphenol A has been found to have endocrine effects (Allard, 2014; Miyagawa *et al*., 2016), and dibutyl phthalate may have endocrine activity (CDC, 2009). The tests undertaken here assessed the effect of the plasticisers on enzyme systems that control acetylcholine management and lipid oxidation, and so do not reflect directly on endocrine action, although it may contribute to changes in the enzyme systems assessed.

Microplastics were found in all samples that are reported on here, illustrating their ubiquity in the rivers that were sampled. The concentrations of microplastics found were fairly high, both in comparison with other sites in South Africa (Bouwman *et al*., 2018), or elsewhere in the world (Li *et al*., 2018; Luo *et al*., 2019; Stanton *et al*., 2020). High microplastic levels accord with the recent observation that plastic consumption is high and plastic waste management in South Africa is failing (Zadan and de Kock, 2020).

This observation raises the question of what such high levels of microplastics might mean for river biota. In the microplastic toxicology exposures undertaken in this project, these field concentrations were relatively low, and in laboratory exposures no toxic impact was detected on a range of endpoints. These results accord with international observations that microplastic exposures that elicit a biotic response are often at levels of microplastics far higher than are encountered in the environment (Triebskorn *et al*., 2019; GESAMP, 2016; Tang, 2017; Lenz *et al*., 2016). In a similar light, European scientific advisers concluded that no known risks were posed by environmental levels of microplastics (SAPEA, 2019).

It is therefore possible that the physical effect of this level of microplastic particles on biota in the environment may be limited or undetectable. However, plasticiser impacts do occur and it would not be wise to write off environmental microplastic levels that are high on a global basis as being safe.

The outcome of the various laboratory and field experiments that were performed as part of the project have helped to achieve the main objectives of this project, which include developing biomonitoring and ecotoxicological methods for studying microplastics in South Africa freshwater systems. The methodological approaches use in the various experimental works can, therefore, be applied as bases

for carrying out similar experiments involving microplastics and plasticisers with respect to their impacts on freshwater systems in South Africa.

## 6.2 RECOMMENDATIONS FOR POLICY AND IMPLEMENTATION

Microplastics pollution is one of the most pervasive emerging environmental issues that confronts freshwater resources in South Africa. The country's freshwater ecosystems are increasingly contaminated with tiny plastic fragments, particles, and fibres, increasingly raising concerns over environmental and human health impacts associated to exposure to these pollutants. The potential for long-term and irreversible risks to ecosystems and human health calls for mitigation measures to be taken to halt the accumulation of plastics and microplastics in freshwater systems of South Africa.

The various studies carried out in this project suggest that potential ecotoxicological effects exist for impact microplastics on freshwater biota and humans due to dependence on freshwater ecosystems. It is therefore important that routine studies are conducted to forestall adverse impacts that might be associated with the use of freshwater resources. This then calls for the application of the standard method, which was, in part, the aim of this project. Thus, this project has developed ecotoxicological and biomonitoring methods that can be used in South Africa when studying microplastics in freshwater. These methods include (1) Sourcing microplastics for toxicity tests: The project provided sources available for supplying microplastics for ecotoxicological experiments. Advice is given as to whether virgin or weathered microplastics are to be tested, what shapes and size ranges can be produced, and the potential for production of these at an industrial and laboratory scale is given. (2) Concentrations and units: The project provided a meaningful way of expressing the concentration of microplastics, and what might be adopted to given ecologically meaningful units. (3) Experimental design: This project considered types of tests that will be most appropriate for assessing physical microplastic effects, test design including consideration of controls, treatments, replication, test concentrations, test taxa, and statistical approaches.

Although the results of the present study seem to suggest that microplastics posed minimal ecological risk at least to the biota tested at concentrations reported in the environment, policy instruments should be directed towards minimizing the entry of plastics materials into freshwater resources. Both punitive and incentive-based systems can be implemented to prevent and/or minimize plastic pollution of freshwater systems. Such policy instruments should consider the plastic value and production chains, as well as behavioral and technological driver of change towards plastic reduction in the environment.

Despite the empirical evidence suggesting the presence of microplastics in South African riverine systems, their occurrence and distribution as well as potential toxicity on indigenous species are poorly studied. A multidisciplinary microplastic monitoring network and programme is recommended. Such a programme would seek to generate data on their occurrence, geospatial distribution, use, toxicity, and human and ecological risk occasioned by microplastics. Such data would be critical for evidence-based policy instruments in South Africa.

Policy instruments should target both hard and soft measures, such as behavioural change, social learning, and technological innovations for the recycling, re-use and reduction of plastic materials through the principles of circular and green economy.

The outcomes of this project could be use by relevant authorities to promote education, research and the application of knowledge in waste management, disposal and development of ecologically acceptable principles and practice in ecosystem management. Cooperation between scientists and

engineers is necessary for designing small scale experimental systems such as for sourcing and processing plastic waste for cost effective housing materials for rural communities and other usable products. Prototypes coming from such collaboration could be regarded as shared intellectual property.

Major contributors to plastics and microplastic pollution include food and beverages, bottle and container caps, plastic bags, straws and stirrers, beverage bottles and containers. Policy guidance is therefore needed to support policymakers looking to comprehensively reduce the leakage of plastics into the environment. From a policy perspective, this project recommends a comprehensive and lifecycle approach to the issue of microplastics pollution. Application of preventive options early in the lifecycle industrial production and usage of plastics and microplastics is will aid pollution mitigation in the most cost-effective manner.

## 6.3 RECOMMENDATIONS FOR FUTURE RESEARCH

The following are recommended for future studies

- An ecological functional approach to toxicity study of microplastic is recommended. This could include an analysis of the effects of microplastics on feeding efficiency, feeding behavior, oxygen uptake and metabolic function. It is likely that effects could be observed if a functional approach is followed.

- Microplastics occurrence and distribution in the riverine systems is potentially mediated by hydrology, hydraulics characteristics and microplastic movements (lateral, vertical and horizontal). A mechanistic approach that seeks to understand the influences of hydrology, and hydraulics on the distribution of microplastics and thus the potential exposure of riverine organisms is recommended.

# REFERENCES


1. Abbasi S, Soltani N, Keshavarzi B, Moore F, Turner A and Hassanaghaei M (2018) Microplastics in different tissues of fish and prawn from the Musa Estuary, Persian Gulf. *Chemosphere* 205: 80-87.
2. Abdel-Tawwab M and Hamed HS (2018) Effect of bisphenol A toxicity on growth performance, biochemical variables and oxidative stress biomarkers of Nile Tilapia, *Oreochromis niloticus* (L.). *Journal of Applied Ichthyology* 34(5): 1117-1125.
3. Abduro Ogo, Hirpa, Na Tang, Xiaowei Li, Xueyuan Gao, and Wei Xing. 2022. “Combined Toxicity of Microplastic and Lead on Submerged Macrophytes.” *Chemosphere* 295(February):133956. doi: 10.1016/j.chemosphere.2022.133956.
4. Adam, Issahaku, Tony R. Walker, Joana Carlos, and Andrea Clayton. 2020. “Policies to Reduce Single-Use Plastic Marine Pollution in West Africa.” *Marine Policy* 116(June 2019):103928. doi: 10.1016/j.marpol.2020.103928.
5. Agency for Toxic Substances and Disease Registry (ATSDR) (2019) ToxFAQs for Di-n-butyl phthalate. https://www.atsdr.cdc.gov/toxfaqs/tf.asp?id=858&tid=167 (accessed on 23 Nov. 2019).
6. Alimi OS, Budarz JF, Hernandez LM and Tufenkji N (2018) Microplastics and Nanoplastics in Aquatic Environments: Aggregation, Deposition, and Enhanced Contaminant Transport. *Environmental Science and Technology* 52: 1704-1724.
7. Allard P (2014) Bisphenol A. In: Biomarkers in Toxicology, ed. Gupta RC. Elsevier, Amsterdam. Andrady, Anthony L. 2011. “Microplastics in the Marine Environment.” *Marine Pollution Bulletin* 62(8):1596-1605. doi: 10.1016/j.marpolbul.2011.05.030.
8. Angnunavuri, Prosper Naah, Francis Attiogbe, Andrew Dansie, and Bismark Mensah. 2020. “Consideration of Emerging Environmental Contaminants in Africa: Review of Occurrence, Formation, Fate, and Toxicity of Plastic Particles.” *Scientific African* 9:e00546. doi: 10.1016/j.sciaf.2020.e00546.
9. Allen AS, Seymour AC, and Rittschof D (2017) Chemoreception drives plastic consumption in a hard coral. *Marine Pollution Bulletin* 124:198-205.
10. Anbumani A and Kakkar P (2018) Ecotoxicological effects of microplastics on biota: a review. *Environmental Science and Pollution Research* 25:14373-14396.
11. Arias-Andres M, Klümper U, Rojas-Jimenez K and Grossart H-P (2018) Microplastic pollution increases gene exchange in aquatic ecosystems. *Environmental Pollution* 237: 253-261.
12. Ašmonaitė G, Sundh H, Asker N and Almroth BC (2018) Rainbow Trout Maintain Intestinal Transport and Barrier Functions Following Exposure to Polystyrene Microplastics. *Environmental Science and Technology* 52: 14392−14401.
13. Au SY, Bruce TF, Bridges WC, Klaine SJ (2015) Responses of *Hyalella azteca* to acute and chronic microplastic exposures. *Environmental Toxicology and Chemistry* 34( 11): 2564-2572.
14. Avio CG, Gorbi S and Regoli F (2017) Plastics and microplastics in the oceans: From emerging pollutants to emerged threat. *Marine Environmental Research* 128: 2-11.
15. Avio CG, Gorbi S, Milan M, Benedetti M, Fattorini D, d'Errico G, Pauletto M, Bargelloni L and Regoli F. (2015). Pollutants bioavailability and toxicological risk from microplastics to marine mussels. *Environmental Pollution* 198: 211-222.
16. Avio GC, Gorbi S and Regoli F (2015) Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: First observations in commercial species from the Adriatic Sea. *Marine Environmental Research* 111: 18-26.
17. Bajka BH, Rigby NM, Cross KL, Macierzanka A and Mackie AR (2015) The influence of small intestinal mucus structure on particle transport ex vivo. *Colloids and Surfaces B Biointerfaces* 135: 73-80.
18. Barboza LGA, Vethaak AD, Lavorant BRBO, Lundebye AK and Guilhermino L (2018c) Marine microplastic debris: An emerging issue for food security, food safety and human health. *Marine Pollution Bulletin* 133: 336-348.
19. Barboza L-GA, Vieira LR and Guilhermino L (2018b) Single and combined effects of microplastics and mercury on juveniles of the European seabass (*Dicentrarchus labrax*): Changes in behavioural responses and reduction of swimming velocity and resistance time. *Environmental Pollution* 236: 1014-1019.
20. Barboza LGA, Vieira LR, Branco V, Figueiredo N, Carvalho F, Carvalho C and Guilhermino L. (2018a). Microplastics cause neurotoxicity, oxidative damage, and energy-related changes and

interact with the bioaccumulation of mercury in the European seabass, *Dicentrarchus labrax* (Linnaeus, 1758). *Aquatic Toxicology* 195: 49-57.
21. Bates DM and Watts DG (1988) *Nonlinear Regression Analysis and Its Applications*. Wiley and Sons, New York.
22. Bayo J, Olmos S and López-Castellanos J (2020) Microplastics in an urban wastewater treatment plant: The influence of physicochemical parameters and environmental factors. *Chemosphere* 238: 124593.
23. Bayo J, Olmos S and López-Castellanos J (2021) Assessment of Microplastics in a Municipal Wastewater Treatment Plant with Tertiary Treatment: Removal Efficiencies and Loading per Day into the Environment. *Water* 13: 1339.
24. Berglund E, Fogelberg V, Nilsson PA and Hollander J (2019) Microplastics in a freshwater mussel (*Anodonta anatina*) in Northern Europe. *Science of the Total Environment* 697:134192.
25. Bertoli, Marco, Paolo Pastorino, Davide Lesa, Monia Renzi, Serena Anselmi, Marino Prearo, and Elisabetta Pizzul. 2022. "Microplastics Accumulation in Functional Feeding Guilds and Functional Habit Groups of Freshwater Macrobenthic Invertebrates: Novel Insights in a Riverine Ecosystem." *Science of the Total Environment* 804:150207. doi: 10.1016/j.scitotenv.2021.150207.
26. Bergmann M, Mützel S, Primpke S, Tekman MB, Trachsel J and Gerdts G (2019) White and wonderful? Microplastics prevail in snow from the Alps to the Arctic. *Science Advances* 5: eaax1157.
27. Besseling E, Wang B, Lürling M, and Koelmans AA. (2014). Nanoplastic Affects the Growth of *S. obliquus* and Reproduction of *D. magna*. *Environmental Science and Technology* 48(20): 12336-12343.
28. Bhattacharya P, Lin S, Turner JP and Ke PC (2010) Physical adsorption of charged plastic nanoparticles affects algal photosynthesis. *Journal of Physical Chemistry C* 114: 16556-16561.
29. Biginagwa FS, Mayoma BS, Shashoua Y, Syberg K and Khan FR (2016) First evidence of microplastics in the African Great Lakes: Recovery from Lake Victoria Nile perch and Nile tilapia. *Journal of Great Lakes Research* 42(1): 146-149.
30. Blair RM, Waldron S, Phoenix VR, Gauchotte-Lindsay C (2019) Microscopy and elemental analysis charactetisation of microplastics in sediment of freshwater urban river in Scotland, UK. *Environmental Science and Pollution Research* 26:12491-12504.
31. Blaser MJ (2014) The microbiome revolution*. Journal of Clinical Investigation* 124(10): 4162-4165.
32. Boonnorat, J.,Chiemchaisri, C., Chiemchaisri, C., Yamamoto, K. (2016) Kinetics of phenolic and phthalic acid esters biodegradation in membrane bioreactor (MBR) treating municipal landfill leachate. *Thermodynamics and Kinetics of Emerging Contaminants in the Environment*. Volume 150 Pages 639-649
33. Bošnir J, Puntarić D, Galić A, Škes I, Dijanić T, Klarić M, Grgić M, Čurković M and Šmit Z (2007) Migration of Phthalates from Plastic Containers into Soft Drinks and Mineral Water. Food Technology and Biotechnology 45(1): 91-95.
34. Bouwman H, Minnaar K, Bezuidenhout C and Verster C (2018) *Microplastics in freshwater environments*. WRC Report No.2610/1/18. Water Research Commission, Pretoria.
35. Brennecke D, Duarte B, Paiva F, Caçador I and Canning-Clode J (2016) Microplastics as vector for heavy metal contamination from the marine environment. *Estuarine, Coastal and Shelf Science* 178: 189-195.
36. Brettschneider, D. J., Misovic, A., Schulte-Oehlmann, U., Oetken, M., & Oehlmann, J. (2019). Detection of chemically induced ecotoxicological effects in rivers of the Nidda catchment (Hessen, Germany) and development of an ecotoxicological, Water Framework Directive-compliant assessment system. *Environmental Sciences Europe*, *31*(1), 1-22.
37. Briggs E, de Moura EAB, Furusawa HA, Cotrim MEB, Oguzie EE, Lugao AB (2019) Microplastics; A novel method for surface water sampling and sample extraction in Elechi creek, Rivers state, Nigeria. *The mineral, metals and materials series*. 269-281.
38. Browne MA, Dissanayake A, Galloway TS, Lowe DM and Thompson RC (2008) Ingested Microscopic Plastic Translocates to the Circulatory System of the Mussel, *Mytilus edulis* (L.). *Environmental Science and Technology* 42: 5026-5031.
39. Budavari S (ed.) (1996) *The Merck Index – An Encyclopedia of Chemicals, Drugs, and Biologicals.* Merck and Co., Inc., Whitehouse Station.
40. Bulannga, Rendani B., and Stefan Schmidt. 2022. "Uptake and Accumulation of Microplastic Particles by Two Freshwater Ciliates Isolated from a Local River in South Africa." *Environmental Research* 204(PB):112123. doi: 10.1016/j.envres.2021.112123.

41. Burns EE and Boxall ABA (2018) Microplastics in the Aquatic Environment: Evidence for or Against Adverse Impacts and Major Knowledge Gaps. *Environmental Toxicology and Chemistry* 37(11): 2776-2796.
42. Burton GA (2017) Stressor Exposures Determine Risk: So, Why Do Fellow Scientists Continue To Focus on Superficial Microplastics Risk?. *Environmental Science and Technology* 51: 13515−13516.
43. Cadogan DF and Howick CJ (2000) Plastizers. In (2001) Kirk-Othmer Encyclopedia of Chemical Technology. Ed. Ley C. John Wiley and Sons, New York.
44. Campanale C, Savino I, Pojar I, Massarelli C and Uricchio VF (2020) A Practical overview of methodologies for sampling and analysis of microplastics in riverine environments. *Sustainability* 12(17): 6755.
45. Carr KE, Smyth SH, McCullough MT, Morris JF and Moyes SM (2012) Morphological aspects of interactions between microparticles and mammalian cells: intestinal uptake and onward movement. *Progress in Histochemistry and Cytochemistry* 46(4): 185-252.
46. Carroll WF Jr, Johnson RW, Moore SS and Paradis RA (2011) Poly(Vinyl Chloride). In: *Applied Plastics Engineering Handbook*, ed. Kutz M. Elsevier, Amsterdam.
47. Carson HS, Colbert SL, Kaylor MJ and McDermid KJ (2011) Small plastic debris changes water movement and heat transfer through beach sediments. *Marine Pollution Bulletin* 62, 1708-1713.
48. Carson, Henry S., Magnus S. Nerheim, Katherine A. Carroll, and Marcus Eriksen. 2013. “The Plastic-Associated Microorganisms of the North Pacific Gyre.” *Marine Pollution Bulletin* 75(1-2):126-32. doi: 10.1016/j.marpolbul.2013.07.054.
49. Catarino AI, Macchia V, Sanderson WG, Thompson RC and Henry TB (2018) Low levels of microplastics (MP) in wild mussels indicate that MP ingestion by humans is minimal compared to exposure via household fibres fallout during a meal. *Environmental Pollution* 237: 675-684.
50. Center for Disease Control and Prevention (CDC) (2009) *National Report on Human Exposure to Environmental Chemicals*. U.S. Department of Health and Human Services, Washington.
51. Chen P, Li S, Liu L and Xu N (2015) Long-term effects of binary mixtures of 17α-ethinyl estradiol and dibutyl phthalate in a partial life-cycle test with zebrafish (Danio Rerio). *Environmental Toxicology and Chemistry* 34(3): 518-526.
52. Chen D, Kannan K, Tan H, Zheng Z, Feng Y-L, Wu Y and Widelka M (2016) Bisphenol Analogues Other Than BPA: Environmental Occurrence, Human Exposure, and Toxicity – A Review. *Environmental Science and Technology* 50: 5438−5453.
53. Chen Q, Gundlach M, Yang S, Jiang J, Velki M, Yin D and Hollert H (2017) Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity. *Science of the Total Environment* 584-585: 1022-1031.
54. Chen, Xinyi, Xixi Li, and Yu Li. (2021). “Toxicity Inhibition Strategy of Microplastics to Aquatic Organisms through Molecular Docking, Molecular Dynamics Simulation and Molecular Modification.” *Ecotoxicology and Environmental Safety* 226. doi: 10.1016/j.ecoenv.2021.112870.
55. Choi JS, Jung Y-J, Hong N-H, Hong SH and Park J-W (2018) Toxicological effects of irregularly shaped and spherical microplastics in a marine teleost, the sheepshead minnow (*Cyprinodon variegatus*). *Marine Pollution Bulletin* 129: 231-240.
56. Claessens M, De Meester S, Van Landuyt L, De Clerck K and Janssen CR (2011) Occurrence and distribution of microplastics in marine sediments along the Belgian coast. *Marine Pollution Bulletin* 62: 2199-2204.
57. Cole M and Galloway TS (2015) Ingestion of Nanoplastics and Microplastics by Pacific Oyster Larvae. *Environmental Science and Technology* 49: 14625-14632.
58. Cole M, Lindeque P, Fileman E, Halsband C, Goodhead R, Moger J and Galloway TS (2013) Microplastic Ingestion by Zooplankton. *Environmental Science and Technology* 47: 6646−6655.
59. Cole M, Lindeque P, Halsband C and Galloway TS (2011) Microplastics as contaminants in the marine environment: A review. *Marine Pollution Bulletin* 62: 2588-2597.
60. Collard F, Gilbert B Compère P, Eppe G, Das K, Jauniaux T and Parmentier E (2017) Microplastics in livers of European anchovies (*Engraulis encrasicolus*, L.). *Environmental Pollution* 229: 1000-1005.
61. Cooper DA and Corcoran PL (2010) Effects of mechanical and chemical processes on the degradation of plastic beach debris on the island of Kauai, Hawaii. *Marine Pollution Bulletin* 60: 650-654.
62. Coppock, A., Leeper, T. J., & Mullinix, K. J. (2018). Generalizability of heterogeneous treatment effect estimates across samples. *Proceedings of the National Academy of Sciences*, *115*(49), 12441-12446

63. Coppock RL, Cole M, Lindeque PK, Queirós AM and Galloway TS (2017) A small-scale, portable method for extracting microplastics from marine sediments. *Environmental Pollution* 230: 829-837.
64. Corcoran PL, Biesinger MC and Grifi M (2009) Plastics and beaches: a degrading relationship. *Marine Pollution Bulletin* 58: 80-84.
65. Cox KD, Covernton GA, Davies HL, Dower JF, Juanes F and Dudas SE (2019) Human Consumption of Microplastics. *Environmental Science and Technology* 53: 7068−7074.
66. Coyle C, Creamer RE, Schulte RPO, O'Sullivan L and Jordan P (2016) A Functional Land Management conceptual framework under soil drainage and land use scenarios*. Environmental Science and Policy* 56: 39-48.
67. de Souza Machado AA, Kloas W, Zarfl C, Hempel S and Rillig MC (2018) Microplastics as an emerging threat to terrestrial ecosystems. *Global Change Biology* 24: 1405-1416.
68. Debellefontaine H, Chakchouk M, Foussard JN, Tissot D and Striolo P (1996) Treatment of organic aqueous wastes: wet air oxidation and wet peroxide oxidation. *Environmental Pollution* 92: 155-164.
69. de Carvalho, Aline Reis, Flavien Garcia, Louna Riem-Galliano, Loïc Tudesque, Magali Albignac, Alexandra ter Halle, and Julien Cucherousset. 2021. "Urbanization and Hydrological Conditions Drive the Spatial and Temporal Variability of Microplastic Pollution in the Garonne River." *Science of the Total Environment* 769. doi: 10.1016/j.scitotenv.2020.144479.
70. Détrée C and Gallardo-Escárate C (2018) Single and repetitive microplastics exposures induce immune system modulation and homeostasis alteration in the edible mussel *Mytilus galloprovincialis*. *Fish and Shellfish Immunology* 83: 52-60.
71. Ding J, Zhang S, Razanajatovo RM, Zou H and Zhu W (2018) Accumulation, tissue distribution, and biochemical effects of polystyrene microplastics in the freshwater fish red tilapia (*Oreochromis niloticus). Environmental Pollution* 238: 1-9.
72. Domínguez-Morueco, N., González-Alonso, S., Valcárcel, Y.,(2014). Phthalate occurrence in rivers and tap water from central Spain. *Science of The Total Environment.* Volumes 500-501, 1 December 2014, Pages 139-146
73. Dorn, P. B., Chou, C. S., & Gentempo, J. J. (1987). Degradation of bisphenol A in natural waters. *Chemosphere*, *16*(7), 1501-1507.
74. Dris R, Gasperi J and Tassin B (2018) Sources and Fate of Microplastics in Urban Areas: A Focus on Paris Megacity. In: Freshwater Microplastics. Handbook of Environmental Chemistry 58. Eds Wagner M and Lambert S. Springer International Publishing, Cham.
75. Dris R, Gasperi J, Mirande C, Mandin C, Guerrouache M, Langlois V and Tassin B (2017) A first overview of textile fibers, including microplastics, in indoor and outdoor environments. *Environmental Pollution* 221: 453-458.
76. Dris R, Gasperi J, Saad M, Mirande C and Tassin B (2016) Synthetic fibers in atmospheric fallout: A source of microplastics in the environment? *Marine Pollution Bulletin* 104: 290-293.
77. Duis K and Coors A (2016) Microplastics in the aquatic and terrestrial environment: sources (with a specific focus on personal care products), fate and effects. *Environmental Sciences Europe* 28: 2.
78. Edjere O, Asibor IG and Otolo SE (2016) Evaluation of the levels of phthalate ester plasticisers in surface water of Ethiope River system, Delta State, Nigeria. *Journal of Applied Sciences and Environmental Management* 20(3): 608-614.
79. Eerkes-Medrano D, Thompson RC and Aldridge DC (2015) Microplastics in freshwater systems: A review of the emerging threats, identification of knowledge gaps and prioritisation of research needs. *Water Research* 75: 63-82.
80. Eldridge JH, Meulbroek JA, Staas JK, Tice TR and Gilley RM (1989) Vaccine-containing biodegradable microspheres specifically enter the gut-associated lymphoid tissue following oral administration and induce a disseminated mucosal immune response. *Advances in Experimental Medicine and Biology* 251: 191-202.
81. Eriksen M, Liboiron M and Kiessling T (2017) Microplastic sampling with the AVANI trawl compared to two neuston trawls in the Bay of Bengal and South Pacific. *Environmental Pollution* 232: 430-439.
82. Eriksen, Tor E., John E. Brittain, Geir Søli, Dean Jacobsen, Peter Goethals, and Nikolai Friberg. 2021. "A Global Perspective on the Application of Riverine Macroinvertebrates as Biological Indicators in Africa, South-Central America, Mexico and Southern Asia." *Ecological Indicators* 126(107609). doi: 10.1016/j.ecolind.2021.107609.

83. Erythropel HC, Maric M, Nicell JA, Leask RL and Yargeau V (2014) Leaching of the plasticiser di(2-ethylhexyl)phthalate (DEHP) from plastic containers and the question of human exposure. *Applied Microbiology and Biotechnology* 98(24): 9967-9981.
84. Espinosa C, Cuesta A and Angeles Esteban M (2017) Effects of dietary polyvinylchloride microparticles on general health, immune status and expression of several genes related to stress in gilthead seabream (*Sparus aurata* L. *Fish and Shellfish Immunology* 68: 251e259.
85. European Chemicals Agency (ECHA) (2019) *Calcium stearate.* https://echa.europa.eu/registration-dossier/-/registered-dossier/18930/5/3/1 (accessed 25 Nov. 2019).
86. European Food Safety Authority (EFSA) (2016) Presence of microplastics and nanoplastics in food, with particular focus on seafood*. EFSA Journal* 14(6):4501.
87. European Union (EU) (2003) *European Union Risk Assessment Report. Bisphenol A, CAS No: 80-05-7.* Institute for Health and Consumer Protection, European Chemicals Bureau, European Commission Joint Research Centre, 3rd Priority List. Office for Official Publications of the European Communities, Luxembourg.
88. Fackelmann G and Sommer S (2019) Microplastics and the gut microbiome: How chronically exposed species may suffer from gut dysbiosis. *Marine Pollution Bulletin* 143: 193-203.
89. Fan, Chihhao, Ya Zhen Huang, Jhen Nan Lin, and Junwei Li. 2021. "Microplastic Constituent Identification from Admixtures by Fourier-Transform Infrared (FTIR) Spectroscopy: The Use of Polyethylene Terephthalate (PET), Polyethylene (PE), Polypropylene (PP), Polyvinyl Chloride (PVC) and Nylon (NY) as the Model Constituent." *Environmental Technology and Innovation* 23:101798. doi: 10.1016/j.eti.2021.101798.
90. Fatoki OS, Bornman M, Ravandhalala L, Chimuka L, Genthe B and Adeniyi A (2010) Phthalate ester plasticisers in freshwater systems of Venda, South Africa and potential health effects. *Water SA* 36(1): 117-125.
91. Fatoki OS and Noma A (2001) Determination of Phthalate Esters in the Aquatic Environment. *South African Journal of Chemistry* 54: 69-83.
92. Fatoki OS and Vernon F (1990) Phthalate Esters in Rivers of the greater Manchester area, U.K. *The Science of the Total Environment* 95: 227-232.
93. Feng P, Ding H, Lin H and Chen W (2017) AOD: the antioxidant protein database. *Nature Scientific Reports* 7: 7449.
94. Ferreira, P., Fonte, E., Guilhermino, L.(2016).Temperature rise and microplastics interact with the toxicity of the antibiotic cefalexin to juveniles of the common goby (*Pomatoschistus microps*): Post-exposure predatory behaviour, acetylcholinesterase activity and lipid peroxidation. *Aquatic Toxicology.* Volume 180, November 2016, Pages 173-185
95. Fox, D. R., & Landis, W. G. (2016). Don't be fooled – A no-observed-effect concentration is no substitute for a poor concentration-response experiment. *Environmental Toxicology and Chemistry*, *35*(9), 2141-2148.
96. Fox J and Weisberg S (2019) *An {R} Companion to Applied Regression, Third Edition*. Thousand Oaks, CA: Sage. URL: https://socialsciences.mcmaster.ca/jfox/Books/Companion/.
97. Free, CM., Jensen, OP., Mason, SA., Eriksen, M., Williamson, NJ., Boldgiv, B. (2014). High-levels of microplastic pollution in a large, remote, mountain lake. Pages 156-163. Volume 85, Issue 1
98. Fries E, Dekiff JH, Willmeyer J, Nuelle MT, Ebert M and Remy D (2013). Identification of polymer types and additives in marine microplastic particles using pyrolysis-GC/MS and scanning electron microscopy. *Environmental Science: Processes and Impacts* 15: 1949-1956.
99. Fromme H, Küchler T, Otto T, Pilz K, Müller J and Wenzel A (2002) Occurrence of phthalates and bisphenol A and F in the environment. *Water Research* 36: 1429-1438.
100. Galloway TS and Lewis CN (2016) Marine microplastics spell big problems for future generations. *Proceedings of the National Academy of Sciences of the United States of America* 113(9): 2331-2333.
101. Gao D-W and Wen Z-D (2016) Phthalate esters in the environment: A critical review of their occurrence, biodegradation, and removal during wastewater treatment processes. *Science of the Total Environment* 541: 986-1001.
102. Garcia JM and Robertson ML (2017) The future of plastics recycling. *Science* 358 (6365): 870-872.
103. Gasperi J, Wright SL, Dris R, Collard F, Mandin C, Guerrouache M, Langlois V, Kelly FJ and Tassin B (2018) Microplastics in air: Are we breathing it in? *Current Opinion in Environmental Science and Health* 1: 1-5.

104. GESAMP (2015) Sources, fate and effects of microplastics in the marine environment: a global assessment. Kershaw PJ (ed). Rep Stud GESAMP No. 90, 96 pp
105. Geyer R, Jambeck JR and Law KL (2017) Production, use, and fate of all plastics ever made. *Science Advances* 3: e1700782.
106. Green, J. W., Springer, T. A., & Holbech, H. (2018). *Statistical analysis of ecotoxicity studies*. John Wiley & Sons.
107. Gobas APC, Otton SV, Tupper-ring LF, Crawford MA, Clark KE and Ikonomou MG (2017) Chemical activity-based environmental risk analysis of the plasticiser di-ethylhexyl phthalate and its main metabolite mono ethylhexyl phthalate. *Environmental Toxicology and Chemistry* 36(6): 1483-1492.
108. Godwin AD (2011) Plasticisers. In: Applied Plastics Engineering Handbook, ed. Kutz M. Elsevier, Amsterdam.
109. Gomiero A, Strafella P and Fabi G (2019) From Macroplastic to Microplastic Litter: Occurrence, Composition, Source Identification and Interaction with Aquatic Organisms. Experiences from the Adriatic Sea. In: Gomiero A (Ed.), *Plastics in the Environment* (pp. 1 20). Intechopen, London.
110. Griffin NJ, Mgaba N, Odume N, Mensah P and Mtintsilana Z (2020) *Deliverable 4: Final report on ecotoxicity testing of microplastics as chemical stressors*. Report on WRC Project K5/2919, submitted to the WRC.
111. Gugliandolo, Enrico, Patrizia Licata, Rosalia Crupi, Ambrogina Albergamo, Amel Jebara, Vincenzo Lo Turco, Angela Giorgia Potortì, Hedi Ben Mansour, Salvatore Cuzzocrea, and Giuseppa Di Bella. 2020. “Plasticisers as Microplastics Tracers in Tunisian Marine Environment.” *Frontiers in Marine Science* 7(December). doi: 10.3389/fmars.2020.589398.
112. Guilhermino L, Vieira LR, Ribeiro G, Tavares AS, Cardoso V, Alves A and Almeida JM (2018) Uptake and effects of the antimicrobial florfenicol, microplastics and their mixtures on freshwater exotic invasive bivalve *Corbicula fluminea*. *Science of the Total Environment* 622-623: 1131-1142.
113. Gutow L, Eckerlebe A, Giménez L and Saborowski R (2016) Experimental Evaluation of Seaweeds as a Vector for Microplastics into Marine Food Webs. *Environmental Science and Technology* 50(2): 915-923.
114. Hahladakis, J. N., Velis, C. A., Weber, R., Iacovidou, E., & Purnell, P. (2018). An overview of chemical additives present in plastics: Migration, release, fate and environmental impact during their use, disposal and recycling. *Journal of hazardous materials*, *344*, 179-199.
115. Hansen E, Nilsson NH, Lithner D and Lassen C (2013) *Hazardous Substances in Plastic Materials*. Oslo: COWI and the Danish Technological Institute on behalf of The Norwegian Climate and Pollution Agency.
116. Hanvey JS, Lewis PJ, Lavers JL, Crosbie ND, Pozode K and Clarke BO (2017) A review of analytical techniques for quantifying microplastics in sediments. *Analytic Methods* 9: 1369-1383.
117. Hermandez E, Nowack B and Mitrano DM (2017) Synthetic textiles as sources of microplastics from household. A mechanistic study to understand microfiber release during washing. *Environmental Science and Technology* 51: 7036-7046.
118. Hernandez LM, Xu EG, Larsson HCE, Tahara R, Maisuria VB and Tufenkji N (2019) Plastic Teabags Release Billions of Microparticles and Nanoparticles into Tea. *Environmental Science and Technology* 53(21): 12300-12310.
119. Horn O, Nalli S, Cooper D and Nicell J (2004) Plasticiser metabolites in the environment. *Water Research* 38: 3693-3698.
120. Horton, A. A., Walton, A., Spurgeon, D. J., Lahive, E., & Svendsen, C. (2017a). Microplastics in freshwater and terrestrial environments: evaluating the current understanding to identify the knowledge gaps and future research priorities. *Science of the total environment*, *586*, 127-141.
121. Horton, A. A., & Dixon, S. J. (2018). Microplastics: An introduction to environmental transport processes. *Wiley Interdisciplinary Reviews: Water*, *5*(2), e1268.
122. Hu L, Su L, Xue Y, Mu J, Zhu J, Xu J and Shi H (2016) Uptake, accumulation and elimination of polystyrene microspheres in tadpoles of *Xenopus tropicalis*. *Chemosphere* 164: 611-617.
123. Huang YQ, Wong CKC, Zheng JS, Bouwman H, Barra R, Wahlström B, Neretin L and Wong MH (2012) Bisphenol A (BPA) in China: A review of sources, environmental levels, and potential human health impacts. *Environment International* 42: 91-99.
124. Imhof HK, Rusek J, Thiel M, Wolinska J and Laforsch C (2017) Do microplastic particles affect *Daphnia magna* at the morphological, life history and molecular level? *PLoS ONE* 12(11): e0187590.

125. Iñiguez ME, Conesa JA and Fullana A (2017) Microplastics in Spanish Table Salt. *Nature Scientific Reports* 7: 8620.
126. Ivleva NP, Wiesheu AC and Niessner R (2017) Microplastic in an aquatic ecosystem. *Angewandte Chemie International Edition* 56: 1720-1739.
127. Jacobson MS, Kevy SV and Grand RJ (1977) Effects of a plasticiser leached from polyvinyl chloride on the subhuman primate: a consequence of chronic transfusion therapy. *Translational Medicine* 89(5): 1066-1079.
128. Jiang C. Yin L. Wen X, Du C, Wu L, Long Y, Liu Y, Ma Y, Yin Q, Zhou Z and Pan H (2018) Microplastics in sediment and surface water of west Dongting lake and south Dongting lake: Abundance, source and composition. *International Journal Environmental Research and Public Health* 15: 2164.
129. Jin Y, Lu L, Tu W, Luo T and Fu Z (2019) Impacts of polystyrene microplastic on the gut barrier, microbiota and metabolism of mice. *Science of the Total Environment* 649: 308-317.
130. Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP) (2016) *Sources, fate and effects of microplastics in the marine environment: part two of a global assessment.* Eds Kershaw PJ Rochman CM. Stud. GESAMP No. 93. International Maritime Organization, London.
131. Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP) (2019) *Guidelines or the monitoring and assessment of plastic litter and microplastics in the ocean*. Eds Kershaw PJ, Turra A and Galgani F. GESAMP Report No. 99. United Nations Environment Programme, Nairobi.
132. Jovanović, B. (2017). Ingestion of microplastics by fish and its potential consequences from a physical perspective. *Integrated environmental assessment and management*, *13*(3), 510-515.
133. Jovanović B, Gökdağ K, Güven O, Emrec Y, Whitley EM and Kideys AE (2018) Virgin microplastics are not causing imminent harm to fish after dietary exposure. *Marine Pollution Bulletin* 130: 123-131.
134. Ju H, Zhu D and Qiao M (2019) Effects of polyethylene microplastics on the gut microbial community, reproduction and avoidance behaviors of the soil springtail, *Folsomia candida*. *Environmental Pollution* 247: 890-897.
135. Kanaujiya, Dipak Kumar, Selvanayaki Sivashanmugam, and Kannan Pakshirajan. 2022. “Biodegradation and Toxicity Removal of Phthalate Mixture by Gordonia Sp. in a Continuous Stirred Tank Bioreactor System.” *Environmental Technology & Innovation* 26:102324. doi: 10.1016/j.eti.2022.102324.
136. Karami A, Golieskardi A, Choo CK, Larat V, Galloway TS and Salamatinia B (2017) The presence of microplastics in commercial salts from different countries. *Nature Scientific Reports* 7: 46173.
137. Karami A, Romano N, Galloway T and Hamzah H (2016) Virgin microplastics cause toxicity and modulate the impacts of phenanthrene on biomarker responses in African catfish (*Clarias gariepinus*). *Environmental Research* 151: 58-70.
138. Karbalaei S, Hanachi P, Walker TR and Cole M (2018) Occurrence, sources, human health impacts and mitigation of microplastic pollution. *Environmental Science and Pollution Research* 25: 36046-36063.
139. Kastner J, Cooper DG, Marić M, Dodd P and Yargeau V (2012) Aqueous leaching of di-2-ethylhexyl phthalate and “green” plasticisers from poly(vinyl chloride). *Science of the Total Environment* 432: 357-364.
140. Kaya AT, Yurtsevera M and Bayraktar SC (2018) Ubiquitous exposure to microfiber pollution in the air. *European Physical Journal Plus* 133: 488.
141. Kelly FD, Sinclair AJ, Mann NJ, Turner AH, Abedin L and Li D (2001) A stearic acid-rich diet improves thrombogenic and atherogenic risk factor profiles in healthy males. *European Journal of Clinical Nutrition* 55: 88-96.
142. Koelmans AA, Nor NHM, Hermsen E, Kooi M, Mintenig SM and De France J (2019) Microplastics in freshwaters and drinking water: Critical review and assessment of data quality. *Water Research* 155: 410-422.
143. Kolandhasamy P, Su L, Li J, Qu X, Jabeen K and Shi H (2018) Adherence of microplastics to soft tissue of mussels: A novel way to uptake microplastics beyond ingestion. *Science of the Total Environment* 610-611: 635-640.
144. Kosuth M, Mason SA and Wattenberg EV (2018) Anthropogenic contamination of tap water, beer, and sea salt. *PLoS ONE* 13(4): e0194970.
145. Kumar, Manish, Hongyu Chen, Surendra Sarsaiya, Shiyi Qin, Huimin Liu, Mukesh Kumar Awasthi, Sunil Kumar, Lal Singh, Zengqiang Zhang, Nanthi S. Bolan, Ashok Pandey, Sunita

Varjani, and Mohammad J. Taherzadeh. 2021. “Current Research Trends on Micro- and Nano-Plastics as an Emerging Threat to Global Environment: A Review.” *Journal of Hazardous Materials* 409(November 2020):124967. doi: 10.1016/j.jhazmat.2020.124967.
146. Kurniawan, Setyo Budi, Nor Sakinah Mohd Said, Muhammad Fauzul Imron, and Siti Rozaimah Sheikh Abdullah. 2021. “Microplastic Pollution in the Environment: Insights into Emerging Sources and Potential Threats.” *Environmental Technology and Innovation* 23:101790. doi: 10.1016/j.eti.2021.101790.
147. Lagarde F, Olivier O, Zanella M, Daniel P, Hiard S and Caruso A (2016) Microplastic interactions with freshwater microalgae: Heteroaggregation and changes in plastic density appear strongly dependent on polymer type. *Environmental Pollution* 215: 331-339.
148. Lambert S, Scherer C and Wagner M (2017) Ecotoxicity testing of microplastics: considering the heterogeneity of physicochemical properties. *International Environmental Assessment and Management* 13(3): 470-475.
149. Lambert S. and Wagner M (2018) Microplastics are contaminants of emerging concern in freshwater environments: an overview In: *Freshwater Microplastics. Handbook of Environmental Chemistry* 58. Eds Wagner M and Lambert S. Springer International Publishing, Cham.
150. Law BD, Bunn WB and Hesterberg TW (1990) Solubility of polymeric organic fibers and manmade vitreous fibers in Gambles Solution*. Inhalation Toxicology* 2: 321-339.
151. Lee, R., Wong, TY., Sabanayagam, C. (2015). Epidemiology of diabetic retinopathy, diabetic macular edema and related vision loss. *Eye and Vis* 2, 17. https://doi.org/10.1186/s40662-015-0026-2
152. Lehner R, Weder C, Petri-Fink A and Rothen-Rutishauser B (2019) Emergence of Nanoplastic in the Environment and Possible Impact on Human Health. *Environmental Science and Technology* 53: 1748−1765.
153. Lei L, Wu S, Lu S, Liu M, Song Y, Fu Z, Shi H, Raley-Susman KM and He D (2018) Microplastic particles cause intestinal damage and other adverse effects in zebrafish *Danio rerio* and nematode *Caenorhabditis elegans*. *Science of the Total Environment* 619-620: 1-8.
154. Lenth R (2020). *emmeans: Estimated Marginal Means, aka Least-Squares Means*. R package version 1.5.1. https://CRAN.R-project.org/package=emmeans.
155. Lenz R, Enders K and Nielsen TG (2016) Microplastic exposure studies should be environmentally realistic. *Proceedings of the National Academy of Sciences of the United States of America* 113(29): E4121-E4122.
156. Ley C (ed.) (2001) *Kirk-Othmer Encyclopedia of Chemical Technology*. Fourth edition. Volume 1. John Wiley and Sons, New York.
157. Li C, Busquets R and Campos LC (2020) Assessment of microplastics in freshwater systems: A review. *Science of the Total Environment* 707: 135578.
158. Li J, Liu H and Chen P (2018) Microplastics in freshwater systems: A review on occurrence, environmental effects, and methods for microplastics detection. *Water Research* 137: 362-374.
159. Li J, Zhang K and Zhang H (2018) Accumulation, tissue distribution, and biochemical effects of polystyrene microplastics in the freshwater fish red tilapia (*Oreochromis niloticus*). *Environmental Pollution* 237: 460-467.
160. Liebezeit G and Liebezeit E (2013) Non-pollen particulates in honey and sugar. *Food Additives and Contaminants: Part A* 30(12): 2136-2140.
161. Liebezeit G and Liebezeit E (2014) Synthetic particles as contaminants in German beers. *Food Additives and Contaminants: Part A* 31(9): 1574-1578.
162. Liu Y, Guan Y, Yang Z, Cai Z, Mizuno T, Tsuno H, Zhu W and Zhang X (2009) Toxicity of seven phthalate esters to embryonic development of the abalone *Haliotis diversicolor supertexta*. *Ecotoxicology* 18(3): 293-303.
163. Li, Yuejiao, Guangxin Yang, Jun Wang, Lin Lu, Xuan Li, Yuqi Zheng, Zhenzhong Zhang, and Shaoguo Ru. 2022. “Microplastics Increase the Accumulation of Phenanthrene in the Ovaries of Marine Medaka (Oryzias Melastigma) and its Transgenerational Toxicity.” *Journal of Hazardous Materials* 424(PD):127754. doi: 10.1016/j.jhazmat.2021.127754.
164. Lin, Li, Xiong Pan, Sheng Zhang, Danwen Li, Wenliang Zhai, Zhen Wang, Jingxiang Tao, Changqing Mi, Qingyun Li, and John C. Crittenden. 2021. “Distribution and Source of Microplastics in China’s Second Largest Reservoir – Danjiangkou Reservoir.” *Journal of Environmental Sciences (China)* 102:74-84. doi: 10.1016/j.jes.2020.09.018.
165. Liu, Xiaomei, Jingkang Ma, Chengliang Yang, Lan Wang, and Jingchun Tang. 2021. “The Toxicity Effects of Nano/Microplastics on an Antibiotic Producing Strain - Streptomyces

Coelicolor M145." *Science of the Total Environment* 764:142804. doi: 10.1016/j.scitotenv.2020.142804.
166. Lourenço, Pedro M., Catarina Serra-Gonçalves, Joana Lia Ferreira, Teresa Catry, and José P. Granadeiro. 2017. "Plastic and Other Microfibers in Sediments, Macroinvertebrates and Shorebirds from Three Intertidal Wetlands of Southern Europe and West Africa." *Environmental Pollution* 231:123-33. doi: 10.1016/j.envpol.2017.07.103.
167. Lu L, Luo T, Zhao Y, Cai C, Fu Z and Jin Y (2019) Interaction between microplastics and microorganism as well as gut microbiota: A consideration on environmental animal and human health. *Science of the Total Environment* 667: 94-100.
168. Lu Y, Zhang Y, Deng Y, Jiang W, Zhao Y, Geng J, Ding L and Ren H (2016) Uptake and Accumulation of Polystyrene Microplastics in Zebrafish (*Danio rerio*) and Toxic Effects in Liver. *Environmental Science and Technology* 50: 4054-4060.
169. Luo W, Su L, Craig NJ, Du F, Wu C and Shi H (2019) Comparison of microplastic pollution in different water bodies from urban creeks to coastal waters. *Environmental Pollution* 246: 174-182.
170. Lusher AL, Hollman PCH and Mendoza-Hill JJ (FAO) (2017) *Microplastics in fisheries and aquaculture: Status of knowledge on their occurrence and implications for aquatic organisms and food safety.* FAO Fisheries and Aquaculture Technical Paper No. 615. Food and Agriculture Organization of the United Nations, Rome.
171. Lyakurwa DJ (2017) Uptake and effects of microplastic particles in selected marine microalgae species; *Oxyrrhis marina* and *Rhodomonas baltica*. MSc thesis, Norwegian University of Science and Technology, Trondhein.
172. Magni S, Gagné F, André C, Della Torre C, Auclair J, Hanana H, Parenti CC, Bonasoro F and Binelli A (2018) Evaluation of uptake and chronic toxicity of virgin polystyrene microbeads in freshwater zebra mussel *Dreissena polymorpha* (Mollusca: Bivalvia). *Science of the Total Environment* 631-632: 778-788.
173. Mai L, Bao LJ, Shi L, Wong CS and Zeng EY (2018) A review of methods for measuring microplastics in aquatic environments. *Environmental Science and Pollution Research* 25(12): 11319-11332.
174. Martin J, Lusher A, Thompson RC and Morley A (2017) The Deposition and Accumulation of Microplastics in Marine Sediments and Bottom Water from the Irish Continental Shelf. *Nature Scientific Reports* 7: 10772.
175. Masura J, Baker J, Foster G and Arthur C (2014) *Laboratory methods for the analysis of microplastics in the marine environment: recommendations for quantifying synthetic particles in waters and sediments*. NOAA Technical Memorandum NOS-OR&R-48. National Oceanic and Atmospheric Administration, Washington DC.
176. Masura, J., Baker, J., Foster, G., & Arthur, C. (2015). Laboratory Methods for the Analysis of Microplastics in the Marine Environment: Recommendations for quantifying synthetic particles in waters and sediments.
177. Mattsson K, Ekvall MT, Hansson L-A, Linse S, Malmendal A and Cederval T (2015) Altered Behavior, Physiology, and Metabolism in Fish Exposed to Polystyrene Nanoparticles. *Environmental Science and Technology* 49: 553−561.
178. Mattsson, K., Johnson, E. V., Malmendal, A., Linse, S., Hansson, L. A., & Cedervall, T. (2017). Brain damage and behavioural disorders in fish induced by plastic nanoparticles delivered through the food chain. *Scientific reports*, *7*(1), 1-7.
179. McCormick AR, Hoellein TJ, London MG, Hittie J, Scott JW and Kelly JJ (2016) Microplastic in surface waters of urban rivers: concentration, sources, and associated bacterial assemblages. *Ecosphere* 7(11): e01556.
180. Merski JA, Johnson WD, Muzzio M, Lyang N-L and Gaworski CL (2008) Oral Toxicity and Bacterial Mutagenicity Studies with a Spunbond Polyethylene and Polyethylene Terephthalate Polymer Fabric. *International Journal of Toxicology* 27: 387-395.
181. Merga, Lemessa B., Paula E. Redondo-Hasselerharm, Paul J. Van den Brink, and Albert A. Koelmans. 2020. "Distribution of Microplastic and Small Macroplastic Particles across Four Fish Species and Sediment in an African Lake." *Science of the Total Environment* 741:140527. doi: 10.1016/j.scitotenv.2020.140527.
182. Mgaba N, Odume N, Mensah P, Griffin N and Mtintsilana Z (2020) *Deliverable 5: Progress Report 2: Microplastics as physical stressors (methods development).* Report on WRC Project K5/2919, submitted to the WRC.

183. Mgaba N, Griffin NJ and Odume ON (2021) *Microplastics as emerging contaminants: Method development, ecotoxicity testing and biomonitoring in South African water resources.* Report on WRC Project K5/2919, submitted to the WRC.
184. Miao L, Wang P, Hou J, Yao Y, Liu Z, Liu S and Li T (2019) Distinct community structure and microbial functions of biofilms colonizing microplastics. *Science of the Total Environment* 650: 2395-2402.
185. Miao, Lingzhan, Yuxuan Gao, Tanveer M. Adyel, Zongli Huo, Zhilin Liu, Jun Wu, and Jun Hou. 2021. “Effects of Biofilm Colonization on the Sinking of Microplastics in Three Freshwater Environments.” *Journal of Hazardous Materials* 413(December 2020):125370. doi: 10.1016/j.jhazmat.2021.125370.
186. Miyagawa S, Sato T and Iguchi T (2016) Bisphenol A. In: *Handbook of Hormones: Comparative Endocrinology for Basic and Clinical Research*, eds. Takei Y, Ando H and Tsutsui K. Elsevier, Amsterdam.
187. Mizraji R, Ahrendt C, Perez-Venegas D, Vargas J, Pulgar J, Aldana M, Ojeda P, Duarte C and Galbán-Malagón C (2017) Is the feeding type related with the content of microplastics in intertidal fish gut? *Marine Pollution Bulletin* 116(1-2): 498-500.
188. Montuori P, Jover E, Morgantini M, Bayona JM and Triassi M (2008) Assessing human exposure to phthalic acid and phthalate esters from mineral water stored in polyethylene terephthalate and glass bottles. *Food Additives & Contaminants: Part A* 25(4): 511-518.
189. Moseley P (2000) Stress proteins and the immune response. *Immunopharmacology.* 48(3):299-302.
190. Mühlschlegel P, Hauk A, Walter U and Sieber R (2017) Lack of evidence for microplastic contamination in honey. *Food Additives and Contaminants: Part* A 34(11): 1982-1989.
191. Murphy F and Quinn B (2018) The effects of microplastic on freshwater *Hydra attenuata* feeding, morphology and reproduction. *Environmental Pollution* 234: 487-494.
192. Murphy J (2003) *Additives for Plastics Handbook*. Second Edition. Elsevier Advanced Technology, Oxford/ New York.
193. Naidoo, Trishan, Anusha Rajkaran, and Sershen. 2020. “Impacts of Plastic Debris on Biota and Implications for Human Health: A South African Perspective.” *South African Journal of Science* 116(5-6):1-8. doi: 10.17159/sajs.2020/7693.
194. National Center for Biotechnology Information (NCBI) (2019a) *PubChem Database. Calcium stearate, CID=15324*, https://pubchem.ncbi.nlm.nih.gov/compound/Calcium-stearate (accessed on 21 Nov. 2019)
195. National Center for Biotechnology Information (NCBI) (2019b). *PubChem Database. Dibutyl phthalate, CID=3026*, https://pubchem.ncbi.nlm.nih.gov/compound/Dibutyl-phthalate (accessed on 21 Nov. 2019)
196. National Center for Biotechnology Information (NCBI) (2019c) *PubChem Database. Bisphenol A, CID=6623*, https://pubchem.ncbi.nlm.nih.gov/compound/Bisphenol-A (accessed on Nov. 28, 2019)
197. National Center for Biotechnology Information (NCBI) (2019d). *PubChem Database. HSDB : 922*, Source=HSDB, https://pubchem.ncbi.nlm.nih.gov/source/hsdb/922#section=Ecotoxicity-Excerpts-(Complete) (accessed on Dec. 2, 2019).
198. National Center for Biotechnology Information (NCBI) (2019e) *PubChem Database. Acetone, CID=180*, https://pubchem.ncbi.nlm.nih.gov/compound/Acetone (accessed on Dec. 3, 2019).
199. National Institute for Occupational Safety and Health (NIOSH) (2019) *Registry of Toxic Effects of Chemical Substances (RTECS)*, https://www.cdc.gov/niosh-rtecs/WI2DC6C0.html (accessed on 22 Nov. 2019).
200. Nature Nanotechnology (2019) Plastic nanoparticles raise concern because of their potential impact on the environment. However, many questions need to be answered to establish how dangerous they really are. *Nature Nanotechnology* 14: 299.
201. Nel, Holly A., Tatenda Dalu, and Ryan J. Wasserman. 2018. “Sinks and Sources: Assessing Microplastic Abundance in River Sediment and Deposit Feeders in an Austral Temperate Urban River System.” *Science of the Total Environment* 612:950-56. doi: 10.1016/j.scitotenv.2017.08.298.
202. Nshimiyimana, Jean Bosco, Sujan Khadka, Piao Zou, Sanjib Adhikari, Ram Proshad, Alina Thapa, and Li Xiong. 2020. “Study on Biodegradation Kinetics of Di-2-Ethylhexyl Phthalate by Newly Isolated Halotolerant Ochrobactrum Anthropi Strain L1-W.” *BMC Research Notes* 13(1):1-6. doi: 10.1186/s13104-020-05096-0.

203. O'Donovan S, Metre NC, Abel S, Fonseca TG, Carteny CC, Cormier B, Keiter SH and Bebianno MJ (2018) Ecotoxicological effects of chemical contaminants adsorbed to microplastics in the clam *Scrobicularia plana. Frontiers in Marine Science* 5: 143.
204. Oberbeckmann S, Kreikemeyer B and Labrenz M (2018) Environmental factors support the formation of specific bacterial assemblages on microplastics*. Frontiers in Microbiology* 8: 2709.
205. Odume, Oghenekaro Nelson. 2020. "Searching for Urban Pollution Signature and Sensitive Macroinvertebrate Traits and Ecological Preferences in a River in the Eastern Cape of South Africa." *Ecological Indicators* 108:105759. doi: https://doi.org/10.1016/j.ecolind.2019.105759.
206. Odume, ON, and N. Mgaba. 2016. "Statistical Analysis of Macroinvertebrate Community Assemblage Structure in Relation to River Health Assessment of an Urban River, Eastern Cape, South Africa." *Aquatic Ecosystem Health and Management* 4988(May 2017). doi: 10.1080/14634988.2016.1255098.
207. Oehlmann J, Oetken M and Schulte-Oehlmann U (2008) A critical evaluation of the environmental risk assessment for plasticisers in the freshwater environment in Europe, with special emphasis on bisphenol A and endocrine disruption. *Environmental Research* 108: 140-149.
208. Oehlmann J, Schulte-Oehlmann U, Bachmann J, Oetken M, Lutz I, Kloas W and Ternes TA (2006) Bisphenol A induces superfeminization in the ramshorn snail (Gastropoda: Prosobranchia) at environmentally relevant concentrations. *Environmental Health Perspectives* 114(Suppl 1): 127-133.
209. Oehlmann J, Schulte-Oehlmann U, Kloas W, Jagnytsch O, Lutz I, Kusk KO, Wollenberger L, Santos EM, Paull GC, Van Look KJW, Tyler CR (2009) A critical analysis of the biological impacts of plasticisers on wildlife. *Philosophical Transactions of the Royal Society of London B Biological Science* 364(1526): 2047-2062.
210. Ogo, H. A., Tang, N., Li, X., Gao, X., & Xing, W. (2022). Combined toxicity of microplastic and lead on submerged macrophytes. *Chemosphere*, *295*, 133956.
211. Ohoro, Chinemerem Ruth, Abiodun Olagoke Adeniji, Lucy Semerjian, Omobola Oluranti Okoh, and Anthony Ifeanyi Okoh. 2021. "Occurrence and Distribution of Pharmaceuticals in Surface Water and Sediment of Buffalo and Sundays River Estuaries, South Africa and Their Ecological Risk Assessment." *Emerging Contaminants* 7:187-95. doi: 10.1016/j.emcon.2021.09.002.
212. Olujimi OO, Fatoki OS, Odendaal JP and Okonkwo JO (2010) Endocrine disrupting chemicals (phenol and phthalates) in the South African environment: a need for more monitoring. *Water SA* 36(5): 671-682.
213. Olujimi OO, Fatoki OS, Odendaal JP and Daso AP (2012) Chemical monitoring and temporal variation in levels of endocrine disrupting chemicals (priority phenols and phthalate esters) from selected wastewater treatment plant and freshwater systems in Republic of South Africa. *Microchemical Journal* 101: 11-23.
214. Ostle C, Thompson RC, Broughton D, Gregory L, Wootton M and Johns DG (2019) The rise in ocean plastics evidenced from a 60-year time series. *Nature Communications* 10: 1622.
215. Pastorino, Paolo, Elisabetta Pizzul, Marco Bertoli, Serena Anselmi, Manuel Kušće, Vasco Menconi, Marino Prearo, and Monia Renzi. 2021. "First Insights into Plastic and Microplastic Occurrence in Biotic and Abiotic Compartments, and Snow from a High-Mountain Lake (Carnic Alps)." *Chemosphere* 265. doi: 10.1016/j.chemosphere.2020.129121.
216. Paul-Pont I, Lacroix C, González Fernández C, Hégaret H, Lambert C,, Le Goïc N, Frère L, Cassone AL, Sussarellu R, Fabioux C, Guyomarch J, Albentosa M, Huvet A and Soudant P (2016) Exposure of marine mussels *Mytilus* spp. to polystyrene microplastics: Toxicity and influence on fluoranthene bioaccumulation. *Environmental Pollution* 216: 724-737.
217. Pauly JL, Stegmeier SJ, Allaart HA, Cheney RT, Zhang PJ, Mayer AG and Streck RJ (1998) Inhaled cellulosic and plastic fibers found in human lung tissue. *Cancer Epidemiology, Biomarkers and Prevention* 7: 419-428.
218. Pedá C, Caccamo L, Fossi MC, Gai F, Andaloro F, Genovese L, Perdichizzi A, Romeo T and Maricchiolo G (2016) Intestinal alterations in European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) exposed to microplastics: Preliminary results. *Environmental Pollution* 212: 251-256.
219. Peijnenburg WJGM and Struijs J (2006) Occurrence of phthalate esters in the environment of the Netherlands. *Ecotoxicology and Environmental Safety* 63: 204-215.
220. Peter R. S., Wagner J. P., https://onlinelibrary.wiley.com/action/doSearch?ContribAuthorRaw=Schreiner%2C+Peter+R(2015) London Dispersion in Molecular Chemistry—Reconsidering Steric Effects. *A journal of the German chemical society*. Volume54, Issue 42

221. Pimentel JC, Avila R and Lourenço AG (1975) Respiratory disease caused by synthetic fibres: a new occupational disease. *Thorax* 30: 204-219.
222. Prata JC (2018) Airborne microplastics: Consequences to human health? *Environmental Pollution* 234: 115-126.
223. Prata, J. C., da Costa, J. P., Girão, A. V., Lopes, I., Duarte, A. C., & Rocha-Santos, T. (2019). Identifying a quick and efficient method of removing organic matter without damaging microplastic samples. *Science of the total environment*, *686*, 131-139.
224. Prata JC, da Costa JP, Lopes I, Duarte AC and Rocha-Santos T (2019) Effects of microplastics on microalgae populations: A critical review. *Science of the Total Environment* 665: 400-405.
225. Prokíc MD, Radovanovíc TB, Gavríc JP and Faggio C (2019) Ecotoxicological effects of microplastics: Examination of biomarkers, current state and future perspectives. *Trends in Analytical Chemistry* 111: 37-46.
226. Qiao R, Sheng C, Lu Y, Zhang Y, Ren H and Lemos B (2019) Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish. *Science of the Total Environment* 662: 246-253.
227. Qu X, Su L, Li H, Liang M and Shi H (2018) Assessing the relationship between the abundance and properties of microplastics in water and in mussels. *Science of the Total Environment* 621: 679-686.
228. R Core Team (2019, 2020). *R: A language and environment for statistical computing.* R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.
229. Rafiee M, Dargahi L, Eslami A, Beirami E, Jahangiri-rad M, Sabour S and Amereh F (2018) Neurobehavioral assessment of rats exposed to pristine polystyrene nanoplastics upon oral exposure. *Chemosphere* 193: 745-753.
230. Rainieri S, Conlledo N, Larsen BK, Granby K and Barranco A (2018) Combined effects of microplastics and chemical contaminants on the organ toxicity of zebrafish (*Danio rerio*). *Environmental Research* 162: 135-143.
231. Renzi M, Guerranti C and Blašković A (2018) Microplastic contents from maricultured and natural mussels. *Marine Pollution Bulletin* 131: 248-251.
232. Revel M, Châtel A and Mouneyrac C (2018) Micro(nano)plastics: A threat to human health? *Current Opinion in Environmental Science and Health* 1: 17-23.
233. Ribeiro R, Garcia AR, Pereira BP, Fonseca M, Mestre NC, Fonseca TG, Ilharco LM and Bebianno RJ (2017) Microplastics effects in *Scrobicularia plana*. *Marine Pollution Bulletin* 122: 379-391.
234. Rist S and Hartmann NB (2018) Aquatic Ecotoxicity of Microplastics and Nanoplastics: Lessons Learned from Engineered Nanomaterials. In: *Freshwater Microplastics. Handbook of Environmental Chemistry* 58. Eds Wagner M and Lambert S. Springer International Publishing, Cham.
235. Rist S, Almroth BC, Hartmann NB and Karlsson TM (2018) A critical perspective on early communications concerning human health aspects of microplastics. *Science of the Total Environment* 626: 720-726.
236. Ritz C, Baty F, Streibig JC and Gerhard D (2015) Dose-Response Analysis Using R. *PLOS ONE* 10(12): e0146021.
237. Rochman CM, Parnis JM, Browne MA, Serrato S, Reiner EJ, Robson M, Young T, Diamond ML and Teh SJ (2017) Direct and indirect effects of different types of microplastics on freshwater prey (*Corbicula fluminea*) and their predator (*Acipenser transmontanus*). *PLoS ONE* 12(11): e0187664.
238. Rosenkranz P, Chaudhry Q, Stone V and Fernandes TF (2009) A comparison of nanoparticle and fine particle uptake by *Daphnia magna*. *Environmental Toxicology and Chemistry* 28: 2142-2149.
239. Ryan, Peter G. 2020. “The Transport and Fate of Marine Plastics in South Africa and Adjacent Oceans.” *South African Journal of Science* 116(5-6):1-9. doi: 10.17159/sajs.2020/7677.
240. Ryan, Peter G., Lorien Pichegru, Vonica Perold, and Coleen L. Moloney. 2020. “Monitoring Marine Plastics – Will We Know If We Are Making a Difference?” *South African Journal of Science* 116(5-6):1-9. doi: 10.17159/sajs.2020/7678.
241. Sadan Z and De Kock L (2020) *Plastics: Facts and Futures: Moving beyond pollution management towards a circular plastics economy in South Africa*. WWF South Africa, Cape Town.
242. Salaudeen, T., Okoh, O., Agunbiade, F., Okoh, A.(2018).Fate and impact of phthalates in activated sludge treated municipal wastewater on the water bodies in the Eastern Cape, South Africa. *Chemosphere*. Volume 203, Pages 336-344

243. SAPEA, Science Advice for Policy by European Academies (2018). A Scientific Perspective on Microplastics in Nature and Society. Berlin: SAPEA. DOI:https://doi.org/10.26356/microplastics
244. SAPEA, Science Advice for Policy by European Academies, 2019. A Scientific Perspective on Microplastics in Nature and Society. Berlin: SAPEA. 10.26356/microplastics. Available from: https://www.sapea.info/microplastics-launch/
245. Scherer C, Brennholt N, Reifferscheid G and Wagner M (2017) Feeding type and development drive the ingestion of microplastics by freshwater invertebrates. *Nature Scientific Reports* 7: 17006.
246. Schirinzi GF, Pérez-Pomeda I, Sanchís J, Rossini C, Farré M and Barceló D (2017) Cytotoxic effects of commonly used nanomaterials and microplastics on cerebral and epithelial human cells. *Environmental Research* 159: 579-587.
247. Schür C, Rist S, Baun A, Mayer P, Hartmann NB and Wagner M (2019) When Fluorescence Is not a Particle: The Tissue Translocation of Microplastics in *Daphnia magna* Seems an Artifact. *Environmental Toxicology and Chemistry* 38(7): 1495-1503.
248. Schwabl P, Köppel S, Königshofer P, Bucsics T Trauner M, Reiberger T and Liebmann B (2019) Detection of Various Microplastics in Human Stool: A Prospective Case Series. *Annals of Internal Medicine* 171(7).
249. Schymanski D, Goldbeck C, Humpf H-U and Fürst P (2018) Analysis of microplastics in water by micro-Raman spectroscopy: Release of plastic particles from different packaging into mineral water. *Water Research* 129: 154-162.
250. Science Advice for Policy by European Academies (SAPEA) (2019) *A Scientific Perspective on Microplastics in Nature and Society*. SAPEA, Berlin.
251. Setälä O, Fleming-Lehtinen V and Lehtiniemi M (2014) Ingestion and transfer of microplastics in the planktonic food web. *Environmental Pollution* 185: 77-83.
252. Shea, Mk. (2003). Pediatric Exposure and Potential Toxicity of Phthalate Plasticizers *Pediatrics* . https://doi.org/10.1542/peds.111.6.1467 111 (6): 1467-1474.
253. Shreiner AB, Kao JY and Young VB (2015) The gut microbiome in health and in disease. *Current Opinion in Gastroenterology* 31(1): 69-75.
254. Silva-Cavalcanti JS, Silva JDB, de França EJ, de Araújo MCB and Gusmão F (2017) Microplastics ingestion by a common tropical freshwater fishing resource. *Environmental Pollution* 221: 218-226.
255. Sjollema SB, Redondo-Hasselerharm P, Leslie HA, Kraak MHS and Vethaak A (2016) Do plastic particles affect microalgal photosynthesis and growth? *Aquatic Toxicology* 170: 259-261.
256. Slabbert L (2004) *Methods for Direct Estimation of Ecological Effect Potential (DEEEP)*. WRC Report No. 1313/1/04. Water Research Commission, Gezina.
257. Smith M, Love DC, Rochman CM and Neff RA (2018) Microplastics in Seafood and the Implications for Human Health. *Current Environmental Health Reports* 5: 375-386.
258. Stanton T, Johnson M, Nathanail P, MacNaughtan W and Gomes RL (2020) Freshwater microplastic concentrations vary through both space and time. *Environmental Pollution* 263: 114481.
259. Staples CA, Dom PB, Kleck GM, O'Block ST and Harris LR (1998) A review of the environmental fate, effects, and exposures of Bisphenol A. *Chemosphere* 36(10): 2149-2173.
260. Su L, Cai H, Kolandhasamy P, Wu C, Rochman CM and Shi H (2018) Using the Asian clam as an indicator of microplastic pollution in freshwater ecosystems. *Environmental Pollution* 234: 347-355.
261. Sun, Jianqiang, Jing Huang, Anping Zhang, Weiping Liu, and Wenwei Cheng. 2013. "Occurrence of Phthalate Esters in Sediments in Qiantang River, China and Inference with Urbanization and River Flow Regime." *Journal of Hazardous Materials* 248-249(1):142-49. doi: 10.1016/j.jhazmat.2012.12.057.
262. Sussarellu R, Suquet M, Thomas Y, Lambert C, Fabioux C, Pernet MEJ, Le Goïc N, Quillien V, Mingant C, Epelboin Y, Corporeau C, Guyomarch J, Robbens J, Paul-Pont I, Soudant P and Huvet A (2016) Oyster reproduction is affected by exposure to polystyrene microplastics. *Proceedings of the National Academy of Sciences of the United States of America* 113(9): 2430-2435.
263. Tang BL (2017) Commentary: Tissue accumulation of microplastics in mice and biomarker responses suggest widespread health risks of exposure. *Frontiers in Environmental Science* 5: 63.
264. Teuten EL, Rowland SJ, Galloway TS, Thompson RC (2007) Potential for plastics to transport hydrophobic contaminants. *Environmental Science and Technology* 41: 7759-7764.

265. Thompson RC, Olsen Y, Mitchell RP, Davis A, Rowland SJ, John AWG, McGonigle D and Russell AE (2004) Lost at sea: where is all the plastic? *Science* 304: 838.
266. Thurén A and Woin P (1991) Effects of phthalate esters on the locomotor activity of the freshwater amphipod *Gammarus pulex*. *Bulletin of Environmental Contamination and Toxicology* 46(1): 159-166.
267. Tickner J (1999) *The use of Di-2-Ethylhexyl Phthalate in PVC medical devices: exposure, toxicity, and alternatives*. Lowell Centre for Sustainable Production, Lowell.
268. Triebskorn R, Braunbeck T, Grummt T, Hanslik L, Huppertsberg S, Jekel M, Knepper TP, Krais S, Müller YK, Pittroff M, Ruhl AS, Schmieg H, Schür C, Strobel C, Wagner M, Zumbülte N and Köhler HR (2019) Relevance of nano- and microplastics for freshwater ecosystems: A critical review. *Trends in Analytical Chemistry* 110: 375-392.
269. Tuan Tran, Huu, Chitsan Lin, Xuan Thanh Bui, Minh Ky Nguyen, Ngoc Dan Thanh Cao, Hussnain Mukhtar, Hong Giang Hoang, Sunita Varjani, Huu Hao Ngo, and Long D. Nghiem. 2022. "Phthalates in the Environment: Characteristics, Fate and Transport, and Advanced Wastewater Treatment Technologies." *Bioresource Technology* 344(PB):126249. doi: 10.1016/j.biortech.2021.126249.
270. Turra A, Manzano AB, Dias RJS, Mahiques MM, Barbosa L, Balthazar-Silva D and Moreira FT (2014) Three dimensional distribution of plastic pellets in sandy beaches – NCBI , *Scientific Reports* 2014(4): 4435.
271. United Nations Environment Programme (UNEP) (2016) *Marine plastic debris and microplastics – Global lessons and research to inspire action and guide policy change*. United Nations Environment Programme, Nairobi.
272. United Nations Environmental Programme and World Health Organisation (UNEP/WHO) (1996) *Water Quality Monitoring: A Practical Guide to the Design and Implementation of Freshwater Quality Studies and Monitoring Programmes*. Eds Bartram J and Balance R. E&FN Spon, London.
273. United, The, Nations World, and Water Development. 2020. *WWAP (UNESCO World Water Assessment Programme), 2019, United Nations World Water Development Report 2020: Water and Climate Change*.
274. Valic F and Zuskin E (1977) Respiratory-function changes in textile workers exposed to synthetic fibers. *Archives of Environmental Health: An International Journal* 32(6), 283e287.
275. Van Cauwenberghe L and Janssen CR (2014) Microplastics in bivalves cultured for human consumption. *Environmental Pollution* 193: 65-70.
276. Vandenberg LN, Hauser R, Marcus M, Olea N and Welshons WV (2007) Human exposure to bisphenol A (BPA). *Reproductive Toxicology* 24: 139-177.
277. Volkheimer G (1993) Persorption of microparticles. *Pathologe* 14(5): 247-252.
278. van Moos N, Burkhardt-Holm P and Köhler A (2012) Uptake and Effects of Microplastics on Cells and Tissue of the Blue Mussel *Mytilus edulis* L. after an Experimental Exposure. *Environmental Science and Technology* 46: 11327-11335.
279. Wan Z, Wang C, Zhou J, Shen M, Wang X, Fu Z and Jin Y (2019) Effects of polystyrene microplastics on the composition of the microbiome and metabolism in larval zebrafish. *Chemosphere* 217: 646-658.
280. Wang, Jun, Luji Bo, Lina Li, Dejian Wang, Gangcai Chen, Peter Christie, and Ying Teng. 2014. "Occurrence of Phthalate Esters in River Sediments in Areas with Different Land Use Patterns." *Science of the Total Environment* 500-501:113-19. doi: 10.1016/j.scitotenv.2014.08.092.
281. Wang F, Wong CX, Chen D, Lu X, Wang F and Zeng EY (2018) Interaction of toxic chemicals with microplastics: A critical review. *Water Research* 139: 208-219.
282. Wang, Yi Xin, Bin Zhou, Ying Jun Chen, Chong Liu, Li Li Huang, Jia Qiang Liao, Xi Jiang Hu, Wen Qing Lu, Qiang Zeng, and An Pan. 2018. "Thyroid Function, Phthalate Exposure and Semen Quality: Exploring Associations and Mediation Effects in Reproductive-Aged Men." *Environment International* 116(January):278-85. doi: 10.1016/j.envint.2018.04.031
283. Wang Y, Mao Z, Zhang M, Ding G, Sun J, Du M, Liu Q, Cong Y, Jin F, Zhang W and Wang J (2019) The uptake and elimination of polystyrene microplastics by the brine shrimp, *Artemia parthenogenetica*, and its impact on its feeding behavior and intestinal histology. *Chemosphere* 234: 123-131.
284. Wang, Ting, Jialin Wang, Qi Lei, Yaning Zhao, Liqing Wang, and Xianyun Wang. 2021. "Microplastic Pollution in Sophisticated Urban River Systems : Combined Influence of Land-Use Types and Physicochemical Characteristics ☆." *Environmental Pollution* 287(January):117604. doi: 10.1016/j.envpol.2021.117604.

285. Warheit DB, Hart GA, Hesterberg TW, Collins JJ, Dyer WM, Swaen GMH, Castranova V, Soiefer AI and Kennedy GL (2001) Potential pulmonary effects of man-made organic fiber (MMOF) dusts. *Critical Reviews in Toxicology* 31: 697-736.
286. Watts AJR, Lewis C, Goodhead RM, Beckett SJ, Moger J, Tyler CR and Galloway TS (2014) Uptake and Retention of Microplastics by the Shore Crab *Carcinus maenas*. *Environmental Science and Technology* 48: 8823-8830.
287. Watts AJR, Urbina MA, Goodhead R, Moger J, Lewis C and Galloway TS (2016) Effect of Microplastic on the Gills of the Shore Crab *Carcinus maenas*. *Environmental Science and Technology* 50 (10): 5364-5369.
288. Welden NAC and Cowie PR (2016) Environment and gut morphology influence microplastic retention in langoustine, *Nephrops norvegicus*. *Environmental Pollution* 214: 859-865.
289. Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenschein C, Watson CS, Zoeller RT and Belcher SM (2007) In vitro molecular mechanisms of bisphenol A action. *Reproductive Toxicology* 24: 178-98.
290. Wickham H (2007) Reshaping Data with the reshape Package. *Journal of Statistical Software* 21(12): 1-20. URL http://www.jstatsoft.org/v21/i12/.
291. Wickham H (2011) The Split-Apply-Combine Strategy for Data Analysis. *Journal of Statistical Software* 40(1): 1-29. URL http://www.jstatsoft.org/v40/i01/.
292. Wickham H, François R, Henry L and Müller K (2020). *dplyr: A Grammar of Data Manipulation*. R package version 1.0.2. https://CRAN.R-project.org/package=dplyr
293. Windsor, Fredric M., Rosie M. Tilley, Charles R. Tyler, and Steve J. Ormerod. 2018. "Microplastic Ingestion by Riverine Macroinvertebrates." *Science of the Total Environment* 646:68-74. doi: 10.1016/j.scitotenv.2018.07.271.
294. Woodall LC, Sanchez-Vidal A, Canals M, Paterson GLJ, Coppock R, Sleight V, Calafat A, Rogers AD, Narayanaswamy BE and Thompson RC (2014) The deep sea is a major sink for microplastic debris. *Royal Society Open Science* 1: 140317.
295. World Health Organisation (WHO) (2017) *Guidelines for drinking-water quality*, fourth edition incorporating the first addendum. World Health Organization, Geneva.
296. World Health Organization (WHO) (2019) *Microplastics in drinking-water.* World Health Organization, Geneva.
297. Wright SL, Thompson RC and Galloway TS (2013) The physical impacts of microplastics on marine organisms: A review. *Environmental Pollution* 178: 483e492.
298. Wright SL and Kelly FJ (2017) Plastic and Human Health: A Micro Issue? *Environmental Science and Technology* 51: 6634−6647.
299. Wright, S. L., Ulke, J., Font, A., Chan, K. L. A., & Kelly, F. J. (2020). Atmospheric microplastic deposition in an urban environment and an evaluation of transport. *Environment international*, *136*, 105411.
300. Wu, Di, Ting Wang, Jing Wang, Lijuan Jiang, Ying Yin, and Hongyan Guo. 2021. "Size-Dependent Toxic Effects of Polystyrene Microplastic Exposure on Microcystis Aeruginosa Growth and Microcystin Production." *Science of the Total Environment* 761. doi: 10.1016/j.scitotenv.2020.143265.
301. Wypych G (ed.) (2017) *Handbook of plasticisers*. Third edition. Elsevier, Amsterdam.
302. Yang J, Yang J, Wu W, Zhao J and Jiang L (2014) Evidence of polyethylene biodegradation by bacterial strains from the guts of plastics-eating waxworms. *Environmental Science and Technology* 48(23): 13776-13784.
303. Yang Y, Liu G, Song W, Ye C, Lin H, Li Z and Liu W (2019) Plastics in the marine environment are reservoirs for antibiotic and metal resistance genes. *Environment International* 123: 79-86.
304. Yang, J., Yang, j., Wu, W. Zhao, J. and Jiang L (2014) Evidence of polyethylene biodegradation by bacterial strains from the guts of plastics-eating waxworms. *Environmental Science and Technology* 48(23): 13776-13784.
305. Yahaya, Abdulrazaq, Omobola O. Okoh, Foluso O. Agunbiade, and Anthony I. Okoh. 2019. "Occurrence of Phenolic Derivatives in Buffalo River of Eastern Cape South Africa: Exposure Risk Evaluation." *Ecotoxicology and Environmental Safety* 171(August 2018):887-93. doi: 10.1016/j.ecoenv.2019.01.037.
306. Yahaya, Abdulrazaq, Omobola O. Okoh, Anthony I. Okoh, and Abiodun O. Adeniji. 2017. "Occurrences of Organochlorine Pesticides along the Course of the Buffalo River in the Eastern Cape of South Africa and Its Health Implications." *International Journal of Environmental Research and Public Health* 14(11). doi: 10.3390/ijerph14111372.

307. Ye L, Guo J and Ge R-S (2014) Chapter Thirteen – Environmental Pollutants and Hydroxysteroid Dehydrogenases. In: *Vitamins and Hormones* Vol 94, ed. Litwack G. Elsevier, Amsterdam.
308. Yin L, Chen B, Xia B, Shi X and Qu K (2018) Polystyrene microplastics alter the behavior, energy reserve and nutritional composition of marine jacopever (*Sebastes schlegelii*). *Journal of Hazardous Materials* 360: 97-105.
309. Yin, Kai, Yu Wang, Hongjing Zhao, Dongxu Wang, Menghao Guo, Mengyao Mu, Yachen Liu, Xiaopan Nie, Baoying Li, Jingyan Li, and Mingwei Xing. 2021. "A Comparative Review of Microplastics and Nanoplastics: Toxicity Hazards on Digestive, Reproductive and Nervous System." *Science of the Total Environment* 774:145758. doi: 10.1016/j.scitotenv.2021.145758.
310. Yokota K, Waterfield H, Hastings C, Davidson E, Kwietniewski E and Wells B (2017) Finding the missing piece of the aquatic plastic pollution puzzle: Interaction between primary producers and microplastics. *Limnology and Oceanography Letters* 2: 91-104.
311. Yu P, Liu Z, Wu D, Chen M, Lu W and Zhao Y (2018) Accumulation of polystyrene microplastics in juvenile *Eriocheir sinensis* and oxidative stress effects in the liver. *Aquatic Toxicology* 200: 28-36.
312. Zadan, S., Ji, W., Richard R., Koepsel, RR., Murata, H., Alan S. C, Alan J. R. (2020). Bactericidal Specificity and Resistance Profile of Poly(Quaternary Ammonium) Polymers and Protein-Poly(Quaternary Ammonium) Conjugates. *Biomacromolecules.* 18, 8, 2583-2593. https://doi.org/10.1021/acs.biomac.7b00705
313. Zbyszewski M, Corcoran PL and Hockin A (2014) Comparison of the distribution and degradation of plastic debris along shorelines of the Great Lakes, North America. *Great Lakes Research* 40: 288-299.
314. Zeileis A and Hothorn T (2002) Diagnostic Checking in Regression Relationships. *R News* 2(3): 7-10. URL https://CRAN.R-project.org/doc/Rnews/
315. Zettler ER, Mincer TJ and Amaral-Zettler LA (2013) Life in the "plastisphere": microbial communities on plastic marine debris. *Environmental Science and Technology* 47: 7137-7146.
316. Zhang C, Chen X, Wang J and Tan L (2017) Toxic effects of microplastic on marine microalgae *Skeletonema costatum*: Interactions between microplastic and algae. *Environmental Pollution* 220: 1282-1288.
317. Zhang, Ya-Qi, Marianna Lykaki, Marta Markiewicz, Mohammad Taher Alrajoula, Caroline Kraas, and Stefan Stolte. 2022. "Environmental Contamination by Microplastics Originating from Textiles: Emission, Transport, Fate and Toxicity." *Journal of Hazardous Materials* 128453. doi: 10.1016/j.jhazmat.2022.128453.
318. Zhang L, Zhang C, Gao R, Yang R and Song Q (2016) Sequence Based Prediction of Antioxidant Proteins Using a Classifier Selection Strategy. *PLoS ONE* 11(9): e0163274.
319. Zhang X and Chen Z (2014) Observing Phthalate Leaching from Plasticized Polymer Films at the Molecular Level. *Langmuir* 30(17): 4933-4944.
320. Zhao L, Qu M, Wong G and Wang D (2017) Transgenerational toxicity of nanopolystyrene particles in the range of μg $L^{-1}$ in the nematode *Caenorhabditis elegans*. *Environmental Science Nano* 4: 2356.
321. Zhao, Changrong, Tong Xu, Miao He, Kinjal J. Shah, Zhaoyang You, Ting Zhang, and Muhammad Zubair. 2021. "Exploring the Toxicity of the Aged Styrene-Butadiene Rubber Microplastics to Petroleum Hydrocarbon-Degrading Bacteria under Compound Pollution System." *Ecotoxicology and Environmental Safety* 227:112903. doi: 10.1016/j.ecoenv.2021.112903.
322. Zhu B-K, Fang Y-M, Zhu D, Christie P, Ke X and Zhu Y-G (2018) Exposure to nanoplastics disturbs the gut microbiome in the soil oligochaete *Enchytraeus crypticus*. *Environmental Pollution* 239: 408-415.
323. Zhu Y, Hua R, Zhou Y, Li H, Quan S and Yu Y (2016) Chronic exposure to mono-(2-ethylhexyl)-phthalate causes endocrine disruption and reproductive dysfunction in zebrafish. *Environmental Toxicology and Chemistry* 35(8): 2117-2124.
324. Ziajahromi S, Kumar A, Neale PA and Leusch FDL (2017) Impact of Microplastic Beads and Fibers on Waterflea (*Ceriodaphnia dubia*) Survival, Growth, and Reproduction: Implications of Single and Mixture Exposures. *Environmental Science and Technology* 51: 13397-13406.
325. Zuma, Bongumusa Msizi. 2010. "Microbial Ecology of the Buffalo River in Response to Water Quality Changes." (March):252.

# APPENDIX A: WORKSHOP REPORT

**Workshop held on 25 June 2019 at the Sarabi Country Lodge, Kempton Park**

The workshop attracted 30 delegates, excluding the 2 research team members who facilitated the workshop. Delegates were drawn from academia, water sector practitioners, the plastics industry, and the military. The summary of the topic explored and discussion held are thus presented below.

The workshop explores three main areas of research/information gaps regarding microplastics. These are i) global perspective on research on microplastics and microplastics management, ii) the toxicity, threat and risk posed by microplastics and iii) methodological issues on the topic.

**Global perspectives on micro plastics**

The main issues highlighted are listed below:

1. That there seems to be insufficient research on the potential effect of microplastic component on human health.

2. Involvement of local government was also indicated as a potential barrier to any management strategy.

3. The imperative for the plastic industry and other industry to be involved as was indicated as key to the success of dealing with microplastics in the environment.

4. Silo operation, particularly among academics was indicated as a barrier to collaboration and standardization of methods.

5. Regarding human health issues, the fact that some plasticisers and additive could be potentially mutagenic and carcinogenic was indicated, and that at the moment no policy on dealing with the problem seems to exist.

***6.*** It was indicated that more research is needed on the existing properties of microplastics that could potentially pose a threat to the environment. Some of these properties include size, shape, chemical leaching, adsorption of metals and endocrine disruptors, metals and thus microplastics acting as vectors. This then raises the question of what about microplastic in the environment that should actually worry us. To this end, workshop delegates agree that the extent and degree of the issue are not well known, but all that is known is that there might be an issue.

**The toxicity, threat and risk posed by microplastics**

The main issues highlighted are listed below:

1. It was indicated that knowledge of microplastics is insufficient for the development of water quality guidelines and any meaningful risk assessment technique/tool.

2. Toxicity testing would require appropriate endpoints considering the mechanical nature of the effects of microplastics based on size, density and shape as well as microplastic accumulation, physical damages to tissues and organs.

3. The potential for microplastics to carry toxins and the toxicity of such toxins was also indicated as a potential area of research.

4. The need for characterisation of exposure route was indicated as important to understanding the potential effects of microplastics.

5. Possible endpoints, feeding, trophic levels and reproduction.

6. Bioassays- daphnia, larvae, standard test organisms, exposure route.

7. In terms of exposure, the following were highlighted hydrophobic nature of microplastics, attachment to sediments, and exposure medium: sediment vs water, and time vs concentration.

8. Other issues indicated are
   What is found in the environment samples?
   What proportion of plastic is degradable?
   Behaviour, size and polymers of microplastics.
   How do microplastics affect behaviours?
   What is the body response to “invasive” components?
   The role of ecosystem parameters such as water column flow.
   Organismal feeding, their capacity to pass particles, and the likelihood of impaction or blockage.
   What limits the excretion rate?
   Particles size can limit trophic level ingestion, and will limit membrane transfer.
   How does charge affect dispersion, and how does it modify physical or chemical effects?
   Microplastics and potential human health impact. Considerations include:
      Exposure route.
      Type of plasticiser in the polymer.
      Potential for endocrine disruption?
      Historical versus new policy driven polymers.

**Methodological issues**

The main issues highlighted are listed below:

**1.** Sample preparation: it was indicated that about 90-100 l of surface water is usually filtered (the filtration method), which is usually followed by drying, density separation and digestion to remove impurities. Sodium chloride and sodium iodide are often used for density separation.

**2.** On biomonitoring, experience exists on analysing microplastic accumulation in animal tissues.

**3.** Lack of standard operating procedure as indicated as a barrier to interlaboratory studies in South Africa.

4. The need to test a range of methods was indicated, particularly both for academia and industry purposes by using both academic and industry labs, bearing cost effectiveness in mind.

5. Without standardized methods, research has reported different size ranges making it difficult for comparing research results.

6. Smaller sizes tend to pose more threat to living organisms.

7. Quality assurance issues, including costs effectiveness is critical for micro plastic research.

8. From a government/practitioner perspective it was highlighted that any method developed should at least have some of the following features: i) be precise and accurate ii) cost effective, iii) short on-site turnaround time, iv) indicate site specific drivers of microplastics, and v) easily to undertake. Simple: efficiency and replication.

# APPENDIX B RAW REPRODUCTION DATA: FISH EGG HATCHING COUNTS

**Table 1** Egg hatching counts for *Danio rerio* eggs exposed to bisphenol A (out of three eggs at experiment start).

| **Day** | **1** | | | **2** | | | **3** | | | **4** | | | **5** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Concentration ($\mu g.L^{-1}$)** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** |
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| 0.25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 |
| 0.5 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 1 | 1 | 2 | 1 | 2 | 2 | 1 |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 2 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 2 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 2 |

**Table 2** Egg hatching counts for *Danio rerio* eggs exposed to dibutyl phthalate (out of three eggs at experiment start).

| **Day** | **1** | | | **2** | | | **3** | | | **4** | | | **5** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Concentration ($\mu L.L^{-1}$)** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** |
| control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 0 | 2 | 0 |
| 0.000135 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 |
| 0.000269 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 3 |
| 0.000538 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 2 | 1 | 0 | 2 | 2 |
| 0.001076 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 3 |
| 0.002152 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |

**Table 3** Egg hatching counts for *Danio rerio* eggs exposed to calcium stearate (out of three eggs at experiment start).

| **Day** | **1** | | | **2** | | | **3** | | | **4** | | | **5** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Concentration ($mg.L^{-1}$)** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** |
| EDTA control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| 0.625 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 1 | 2 | 1 | 2 | 3 | 1 | 3 |
| 1.2l | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 1 | 3 | 2 | 2 |
| 2.5 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 2 | 1 | 1 | 3 | 1 | 2 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 2 | 1 | 0 | 2 | 1 | 0 |

# APPENDIX C RAW REPRODUCTION DATA: SNAIL OFFSPRING COUNTS

**Table 4** Offspring number from adult *Melania tuberculata* exposed to bisphenol A (from two adults at experiment start).

| **Day** | **1** | | | **5** | | | **10** | | | **15** | | | **21** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Concentration ($\mu g.L^{-1}$)** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** |
| control | 1 | 0 | 0 | 2 | 1 | 0 | 2 | 1 | 2 | 2 | 3 | 3 | 3 | 3 | 3 |
| 0.25 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 3 | 1 | 2 | 3 |
| 0.5 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 2 | 0 | 2 | 3 | 1 | 2 | 3 | 1 |
| 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 1 | 2 | 0 | 1 | 2 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 |

**Table 5** Offspring number from adult *Melania tuberculata* exposed to dibutyl phthalate (from five adults at experiment start). The experiment was terminated before day 21 owing to the Covid lockdown.

| **Day** | **5** | | | **10** | | | **15** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Concentration ($\mu L.L^{-1}$)** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** |
| control | 1 | 0 | 1 | 1 | 0 | 2 | 1 | 0 | 2 |
| 0.000135 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 3 | 1 |
| 0.000269 | 0 | 3 | 1 | 0 | 3 | 2 | 2 | 3 | 2 |
| 0.000538 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 |
| 0.001076 | 0 | 2 | 0 | 0 | 3 | 1 | 0 | 3 | 1 |
| 0.002152 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |

**Table 6** Offspring number from adult *Melania tuberculata* exposed to calcium stearate (from two adults at experiment start).

| **Day** | **1** | | | **5** | | | **10** | | | **15** | | | **21** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Concentration ($mg.L^{-1}$)** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** | **A** | **B** | **C** |
| Control | 1 | 0 | 0 | 2 | 1 | 0 | 2 | 1 | 0 | 2 | 1 | 1 | 2 | 1 | 1 |
| EDTA | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 |
| 0.625 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 2 | 0 | 1 | 2 | 0 | 1 | 2 |
| 1.25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| 2.5 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 2 | 1 | 0 | 2 | 1 | 1 | 2 |
| 5.0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 1 | 2 |

**Table 7** Number of offspring per adult *Melania tuberculata* in three replicates exposed to polypropylene.

| **Day** | **5** | | | **10** | | | **15** | | | **21** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** | **1** | **2** | **3** | **1** | **2** | **3** | **1** | **2** | **3** |
| Control | 0 | 0 | 0 | 0 | 0.5 | 0 | 0.5 | 0.5 | 0 | 0.5 | 0.5 | 0 |
| 1566 | 0.5 | 0 | 0.5 | 1 | 0 | 0.5 | 1.5 | 0 | 1 | 1.5 | 0 | 1 |
| 3131 | 0 | 0 | 0.5 | 0 | 0 | 0.5 | 0 | 0.5 | 0.5 | 0 | 0.5 | 0.5 |
| 6263 | 0 | 0 | 0 | 0 | 0.5 | 0.5 | 0 | 0.5 | 0.5 | 0 | 0.5 | 1 |

**Table 8** Number of offspring per adult *Melania tuberculata* in three replicates exposed to polyethylene.

| **Day** | | **5** | | | **10** | | | **15** | | | **21** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** | **1** | **2** | **3** | **1** | **2** | **3** | **1** | **2** | **3** |
| Control | 0 | 0 | 0 | 0 | 0.5 | 0 | 0.5 | 0.5 | 0 | 0.5 | 0.5 | 0 |
| 923 | 0 | 0 | 0 | 0 | 0.5 | 0 | 0 | 0.5 | 0 | 0 | 0.5 | 0 |
| 1846 | 0.5 | 0 | 0 | 0.5 | 0 | 0 | 0.5 | 0 | 0 | 1 | 0.5 | 0 |
| 3691 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 9** Number of offspring per adult *Melania tuberculata* in three replicates exposed to polyvinyl chloride.

| **Day** | | **5** | | | **10** | | | **15** | | | **21** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** | **1** | **2** | **3** | **1** | **2** | **3** | **1** | **2** | **3** |
| Control | 0 | 0 | 0 | 0 | 0.5 | 0 | 0.5 | 0.5 | 0 | 0.5 | 0.5 | 0 |
| 720 | 0.5 | 0 | 0 | 0.5 | 0 | 0 | 1 | 0 | 0 | 1.5 | 0 | 0 |
| 1440 | 0 | 0 | 0 | 0.5 | 0 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| 2880 | 0 | 0 | 0 | 0 | 0.5 | 0 | 0 | 0.5 | 0.5 | 0 | 0.5 | 0.5 |

# APPENDIX D RAW GROWTH DATA: MELANIA TUBERCULATA SHELL LENGTH

**Table 10** Shell length (mm) of adult *Melania tuberculata* exposed to bisphenol A for 21 days (from two adults at experiment start).

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration ($\mu g.L^{-1}$)** | **A** | **B** | **C** |
| control | 12, 12 | 13, 16 | 12, 14 |
| 0.25 | 14, 12 | 13, 13 | 14, 15.2 |
| 0.5 | 12, 13 | 16, 15.5 | 16, 14.8 |
| 1 | 12, 13 | 14, 12.5 | 13, 12.7 |
| 2 | 13, 12.5 | 12, 12.3 | 13, 12 |
| 4 | 13.5, 15 | 14, 12 | 14, 13.5 |

**Table 11** Shell length (mm) of juvenile *Melania tuberculata* exposed to bisphenol A after 21 days.

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration ($\mu g.L^{-1}$)** | **A** | **B** | **C** |
| control | 3.5, 3.2 | 3.6, 3, 2.3 | 2.5, 3, 1.9 |
| 0.25 | 2.3 | 3, 1.8 | 2, 1.7, 1.8 |
| 0.5 | 2.1, 2.7 | 1.1, 2, 2.1 | 1.8 |
| 1 | 1.8 | 2.2, 2.8 | |
| 2 | | | 2.3 |
| 4 | | | 1.8 |

**Table 12** Shell length (mm) of adult *Melania tuberculata* exposed to dibutyl phthalate for 15 days (from five adults at experiment start).

| **Day** | **15** | | |
|---|---|---|---|
| **Concentration ($\mu L.L^{-1}$)** | **A** | **B** | **C** |
| control | 11, 11.5, 10.5, 12.5, 12.5 | 11, 11.3, 10, 12, 12.5 | 11, 12, 10.2, 12, 12 |
| 0.000135 | 10, 11.5, 10.5, 13.5, 10.5 | 11, 8.5, 10.2, 10.2, 12 | 12, 11.5, 10.5, 11.5, 10 |
| 0.000269 | 11, 11.5, 10.5, 12 | 12, 9, 10, 12, 12 | 11, 8, 10.5, 12, 12 |
| 0.000538 | 11, 10.5, 10, 10.5, 9 | 11, 9, 8, 8, 13 | 11, 9, 10, 8, 12 |
| 0.001076 | 11. , 10, 10, 12, 12 | 11, 8, 10, 13, 12 | 11, 11.5, 7, 13, 13 |
| 0.002152 | 11, 11.5, 7, 10, 11 | 12, 9.5, 12.5, 10, 9.5 | 10.5, 9, 11, 11, 11.5 |

**Table 13** Shell length (mm) of juvenile *Melania tuberculata* exposed to dibutyl phthalate after 21 days.

| **Day** | **15** | | |
|---|---|---|---|
| **Concentration ($\mu L.L^{-1}$)** | **A** | **B** | **C** |
| control | 3 | | 3.2 |
| 0.000135 | 2.5 | 2, 1.5, 2 | 2.5 |
| 0.000269 | 2, 1.5 | 3, 2, 2 | 2.5, 2.5 |
| 0.000538 | 2 | 2 | 3 |
| 0.001076 | | 2.5, 1.5, 3 | 2 |
| 0.002152 | 2.5 | 2.5, 2.5 | 1.5, 2.5, 3 |

**Table 14** Shell length (mm) of adult *Melania tuberculata* exposed to calcium stearate for 21 days (from two adults at experiment start).

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration ($mg.L^{-1}$)** | **A** | **B** | **C** |
| Control | 12, 11.8 | 11, 12.5 | 13, 15 |
| EDTA | 12.5, 11 | 11, 12 | 12, 12 |
| 0.625 | 14, 12 | 14.5, 11 | 12.5, 12 |
| 1.25 | 12.2, 11 | 12, 11.5 | 13, 11.3 |
| 2.5 | 12.3, 13 | 12.2, 11 | 13.5, 12 |
| 5.0 | 12.2, 13 | 11.8, 11.2 | 13.5, 11.3 |

**Table 15** Shell length (mm) of juvenile *Melania tuberculata* exposed to calcium stearate for 21 days.

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration ($mg.L^{-1}$)** | **A** | **B** | **C** |
| Control | 3, 3 | 2.8 | 3 |
| EDTA | 3.2 | 3 | |
| 0.625 | | 2 | 2.1, 2.5 |
| 1.25 | | 2.1 | |
| 2.5 | 3 | 2.5 | 2.5, 3.2 |
| 5.0 | | 2 | 2.5, 3.2 |

**Table** 16 Shell length (mm) in adult *Melania tuberculata* in three replicates exposed to polypropylene for 21 days

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** |
| Control | 3, 2.8 | 3.3, 2.8 | 3.5, 3.1 |
| 1566 | 3, 3.1 | 3.4, 2.5 | 4.8, 4.2 |
| 3131 | 3.1, 2.8 | 3.2, 3 | 2.8, 3.1 |
| 6263 | 3, 2.5 | 3.5, 3 | 4, 2.5 |

**Table** 17 Shell length in juvenile *Melania tuberculata* in three replicates exposed to polypropylene for 21 days.

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** |
| Control | 1.5 | 0.6 | |
| 1566 | 0.5, 0.3, 0.8 | | 0.6, 0.8 |
| 3131 | | 0.8 | 0.5 |
| 6263 | | 1 | 1, 0.7 |

**Table 18** Shell length in adult *Melania tuberculata* in three replicates exposed to polyethylene for 21 days.

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** |
| Control | 3, 2.8 | 3.3, 2.8 | 3.5, 3.1 |
| 923 | 3.3, 3.2 | 3 | 3, 3.4 |
| 1846 | 3.2, 2.5 | 3.5, 3 | 4, 2.8 |
| 3691 | 4, 3 | 3.4, 3 | 3.8, 3.5 |

**Table 19** Shell length in juvenile *Melania tuberculata* in three replicates exposed to polyethylene for 21 days.

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** |
| Control | 1.5 | 0.6 | |
| 923 | | 1 | |
| 1846 | 0.4, 0.5 | 1.3 | |
| 3691 | | | |

**Table 20** Shell length in adult *Melania tuberculata* in three replicates exposed to polyvinyl chloride for 21 days.

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration(particles/L)** | **1** | **2** | **3** |
| Control | 3, 2.8 | 3.3, 2.8 | 3.5, 3.1 |
| 720 | 3, 3.5 | 3.5, 3.5 | 3, 3.1 |
| 1440 | 4, 2.5 | 4, 2 | 3.3, 3 |
| 2880 | 2.8, 3.1 | 3.4, 2.5 | 3, 3, 0.7 |

**Table 21** Shell length in juvenile *Melania tuberculata* in three replicates exposed to polyvinyl chloride for 21 days.

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** |
| Control | 1.5 | 0.6 | |
| 720 | 0.8, 0.4, 1 | | |
| 1440 | 0.7 | 0.8 | 1 |
| 2880 | | 0.4 | |

# APPENDIX E RAW GROWTH DATA: JUVENILE FISH BODY SIZE

**Table 22** Body length (mm) of juvenile *Tilapia sparrmanii* exposed to bisphenol A for 21 days (from two adults at experiment start).

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration ($\mu g.L^{-1}$)** | **A** | **B** | **C** |
| control | 16, 15 | 14, 12 | 14, 17 |
| 0.25 | 13, 14 | 13, 16.5 | 15.5, 15 |
| 1 | 17, 16 | 17, 18 | 16, 15 |
| 4 | 18.5, 17 | 16, 19 | 16, 19 |

**Table 23** Body length (mm) of juvenile *Tilapia sparrmanii* exposed to calcium stearate for 21 days (from two adults at experiment start).

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration ($mg.L^{-1}$)** | **A** | **B** | **C** |
| EDTA | 13, 15 | 14.5, 16.5 | 12, 13 |
| 0.625 | 13, 18 | 14, 14 | 14, 16 |
| 1.25 | 13, 18 | 10, 17 | 15, 16 |
| 5.0 | 13, 13 | 12, 16 | 14, 16 |

**Table 24** Body length (mm) of juvenile *Tilapia sparrmanii* from three replicates exposed to a polypropylene suspension of differing concentrations for 21 days.

| **Day** | **21** | | |
|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** |
| Control | 56, 50 | 55, 52 | 65, 50 |
| 1566 | 50, 52 | 54, 53 | 53, 53 |
| 3132 | 57, 48 | 50.5, 48 | 54, 50 |
| 6264 | 49, 62 | 50.5, 56 | 46, 52 |

**Table 25** Body length (mm) of juvenile *Tilapia sparrmanii* from three replicates exposed to a polyethylene suspension of differing concentrations for 21 days.

| **Day** | **21** | | |
|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** |
| Control | 56, 50 | 55, 52 | 65, 50 |
| 922 | 59, 38 | 61.5, 47 | 58, 50 |
| 1844 | 45, 51 | 49, 54 | 61, 63 |
| 3699 | 47, 49 | 45, 59 | 45, 51 |

**Table 26** Body length (mm) of juvenile *Tilapia sparrmanii* from three replicates exposed to a polyvinyl chloride suspension of differing concentrations for 21 days.

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** |
| Control | 56, 50 | 55, 52 | 65, 50 |
| 721 | 54, 67 | 49, 52 | 55, 47 |
| 1442 | 67, 65 | 65, 49 | 52, 47 |
| 2884 | 65, 56.5 | 48, 51.5 | 52, 48.5 |

**Table 27** Body width (mm) of juvenile *Tilapia sparrmanii* from three replicates exposed to a polypropylene suspension of differing concentrations for 21 days.

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** |
| Control | 19,15 | 17,16 | 22.5,14 |
| 1566 | 15,15 | 16,15 | 17,17 |
| 3132 | 17,14.5 | 15,13.5 | 15.5,13.5 |
| 6264 | 16,18 | 15,17.5 | 13.5,16.5 |

**Table 28** Body width (mm) of juvenile *Tilapia sparrmanii* from three replicates exposed to a polyethylene suspension of differing concentrations for 21 days

| **Day** | **21** | | |
|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** |
| Control | 19,15 | 17,16 | 22.5,14 |
| 922 | 18.5,14.5 | 19,14 | 17.5,15 |
| 1844 | 12.5,16 | 14.5,15 | 21,21.5 |
| 3699 | 14,15.5 | 14.5,20 | 12.5,15 |

**Table 29** Body width (mm) of juvenile *Tilapia sparrmanii* from three replicates exposed to a polyvinyl chloride suspension of differing concentrations for 21 days.

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** |
| Control | 19,15 | 17,16 | 22.5,14 |
| 1566 | 15,15 | 16,15 | 17,17 |
| 3132 | 17,14.5 | 15,13.5 | 15.5,13.5 |
| 6264 | 16,18 | 15,17.5 | 13.5,16.5 |

**Table 30** Body width (mm) of juvenile *Tilapia sparrmanii* from three replicates exposed to a bisphenol A solution of differing concentrations for 21 days

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration (µg/L)** | **1** | **2** | **3** |
| Control | 16,15 | 14,12 | 14,17 |
| 0.25 | 13,14 | 13,16.5 | 15.5,16 |
| 1 | 17,16 | 17,18 | 16,15 |
| 4 | 18.5,17 | 16,19 | 16,19 |

**Table 31** Body width (mm) of juvenile *Tilapia sparrmanii* from three replicates exposed to a dibutyl phthalate solution of differing concentrations for 21 days.

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration (µL.L$^{-1}$)** | **1** | **2** | **3** |
| Control | 16,15 | 14,12 | 14,17 |
| 0.000135 | 14.5,12 | 17,17 | 17,10.5 |
| 0.000538 | 14,17 | 12,16 | 14,17 |
| 0.002152 | 17,16 | 17,16 | 16.5,14 |

Width cas

**Table 32** Body width (mm) of juvenile *Tilapia sparrmanii* from three replicates exposed to a calcium stearate solution of differing concentrations for 21 days.

| **Day** | | **21** | |
|---|---|---|---|
| **Concentration (mg.L$^{-1}$)** | **1** | **2** | **3** |
| Control | 16,15 | 14,12 | 14,17 |
| EDTA | 13,15 | 14.5,16.5 | 12,13 |
| 0.625 | 13,18 | 14,14 | 14,16 |
| 1.25 | 13,18 | 10,17 | 15,16 |
| 5 | 13,13 | 12,16 | 14,16 |

**Table 33** Body mass (g) of juvenile *Tilapia sparrmanii* from three replicates exposed to a polypropylene suspension of differing concentrations for 21 days.

| **Day** | **21** | | |
|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** |
| Control | 3.5,2.4 | 2.7,2.5 | 4.9,2.3 |
| 1566 | 2.5,2.5 | 2.8,3 | 3.2,2.9 |
| 3132 | 3.5,2 | 3.2,2.7 | 2.9,2.8 |
| 6264 | 2.7,5.4 | 3.1,3.8 | 1.9,2.9 |

**Table 34** Body mass (g) of juvenile *Tilapia sparrmanii* from three replicates exposed to a polyethylene suspension of differing concentrations for 21 days.

| **Day** | **21** | | |
|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** |
| Control | 3.5,2.4 | 2.7,2.5 | 4.9,2.3 |
| 922 | 3.6,2.2 | 4.5,2.2 | 3.8,2.4 |
| 1844 | 1.8,2.5 | 2.4,2.7 | 4.9,4.4 |
| 3699 | 1.8,2.5 | 1.7,4.2 | 1.5,2.7 |

**Table 35** Body mass (g) of juvenile *Tilapia sparrmanii* from three replicates exposed to a polyvinyl chloride suspension of differing concentrations for 21 days.

| **Day** | **21** | | |
|---|---|---|---|
| **Concentration (particles/L)** | **1** | **2** | **3** |
| Control | 3.5,2.4 | 2.7,2.5 | 4.9,2.3 |
| 721 | 3.3,5.1 | 2.4,2.1 | 3.3,2 |
| 1442 | 4.7,4.3 | 4.1,1.9 | 2.4,2 |
| 2884 | 3.3,2.5 | 2.1,2.3 | 2,2.4 |

# APPENDIX F RAW GROWTH DATA: JUVENILE SHRIMP CARAPACE LENGTH

**Table 36** Body length (mm) of juvenile *Caridina nilotica* exposed to calcium stearate for 21 days.

| **Day** | **21** | |
|---|---|---|
| **Concentration ($mg.L^{-1}$)** | **A** | **B** |
| Control | 18 | 23 |
| EDTA | 21 | 19 |
| 0.625 | 19 | 20 |
| 1.25 | 20 | 21 |
| 5.0 | 20 | 21 |

**Table 37** Table 20 Body length (mm) of juvenile *Caridina nilotica* exposed to bisphenol A for 21 days.

| **Day** | **21** | |
|---|---|---|
| **Concentration ($\mu g.L^{-1}$)** | **A** | **B** |
| Control | 18 | 23 |
| 0.25 | 22 | 18 |
| 1 | 18 | 16 |
| 4 | 22 | 17 |

# APPENDIX G MICROPLASTIC PARTICLES EGESTED: JUVENILE FISH

**Table 38** Microplastic particles egested by juvenile *Tilapia sparrmanii* housed in a polypropylene suspension for various lengths of time.

| **Day** | | | | | | |
|---|---|---|---|---|---|---|
| **Concentration (particles/L)** | **1** | **5** | **10** | **15** | **20** | **21** |
| 1566 | 1 | 0 | 3 | 1 | 0 | 0 |
| 3132 | 0 | 2 | 1 | 1 | 0 | 0 |
| 6264 | 3 | 0 | 1 | 1 | 1 | 0 |

**Table 39** Microplastic particles egested by juvenile *Tilapia sparrmanii* housed in a polyethylene suspension for various lengths of time.

| **Day** | | | | | | |
|---|---|---|---|---|---|---|
| **Concentration (particles/L)** | **1** | **5** | **10** | **15** | **20** | **21** |
| 922 | 3 | 4 | 2 | 1 | 0 | 1 |
| 1844 | 4 | 2 | 4 | 2 | 2 | 1 |
| 3699 | 3 | 6 | 5 | 2 | 1 | 2 |

**Table 40** Microplastic particles egested by juvenile *Tilapia sparrmanii* housed in a polyvinyl chloride suspension for various lengths of time.

| **Day** | | | | | | |
|---|---|---|---|---|---|---|
| **Concentration (particles/L)** | **1** | **5** | **10** | **15** | **20** | **21** |
| 721 | 3 | 2 | 1 | 0 | 0 | 0 |
| 1442 | 4 | 0 | 3 | 1 | 0 | 0 |
| 2884 | 3 | 3 | 1 | 1 | 1 | 1 |

# APPENDIX H RAW ACETYLCHOLINESTERASE ACTIVITY DATA

**Table 41** Acetylcholinesterase activity per milligram of protein in *Caridina nilotica* juveniles exposed to calcium stearate.

| **Day** | **21** | |
|---|---|---|
| **Concentration (mg/L)** | **1** | **2** |
| Control | 0.009 | 0.006 |
| EDTA | 0.049 | 0.073 |
| 0.625 | 0.012 | 0.097 |
| 1.25 | 0.056 | 0.157 |
| 5.0 | 0.150 | 2.537 |

**Table 42** Acetylcholinesterase activity per milligram of protein in *Caridina nilotica* juveniles exposed to bisphenol A**.**

| **Day** | **21** | |
|---|---|---|
| **Concentration (µg/L)** | **1** | **2** |
| Control | 0.024 | 0.024 |
| 0.25 | 0.423 | 0.202 |
| 1.0 | 0.019 | 0.015 |
| 4.0 | 0.613 | 0.116 |

**Table 43** Acetylcholinesterase activity per milligram of protein in *Caridina nilotica* juveniles exposed to dibutyl phthalate.

| **Day** | **21** | |
|---|---|---|
| **Concentration (nL/L)** | **1** | **2** |
| Control | 0.024 | 0.024 |
| 0.135 | 0.047 | 0.032 |
| 0.53 | 0.052 | 0.027 |
| 2.152 | 0.036 | 0.336 |

# APPENDIX I RAW LIPID PEROXIDASE ACTIVITY DATA

**Table 44** Lipid peroxidase activity per milligram of protein in *Caridina nilotica* juveniles exposed to calcium stearate.

| **Day** | **21** | |
|---|---|---|
| **Concentration (mg/L)** | **1** | **2** |
| Control | 109 | 93 |
| EDTA | 249 | 359 |
| 0.625 | 53 | 83 |
| 1.25 | 113 | 119 |
| 5.0 | 75 | 3683 |

**Table 45** Lipid peroxidase activity per milligram of protein in *Caridina nilotica* juveniles exposed to bisphenol A.

| **Day** | **21** | |
|---|---|---|
| **Concentration (µg/L)** | **1** | **2** |
| Control | 289 | 368 |
| 0.25 | 89 | 157 |
| 1.0 | 143 | 52 |
| 4.0 | 400 | 173 |

**Table 46** Lipid peroxidase activity per milligram of protein in *Caridina nilotica* juveniles exposed to dibutyl phthalate.

| **Day** | **21** | |
|---|---|---|
| **Concentration (nL/L)** | **1** | **2** |
| Control | 289 | 369 |
| 0.135 | 298 | 235 |
| 0.53 | 507 | 229 |
| 2.152 | 1458 | 841 |

# APPENDIX J RAW ENVIRONMENTAL MICROPLASTIC DATA

**Table 47** Microplastic particle concentrations in environmental water samples from the Swatkops and Buffalo River catchments.

| River | Site name | Hydraulic conditions | Date | Particles $L^{-1}$ |
|---|---|---|---|---|
| Swartkops | Swartkops 2 | Pool | 15 Jun 2021 | 8.8 |
| Swartkops | Swartkops 2 | Pool | 5 Feb 2021 | 16.9 |
| Swartkops | Swartkops 2 | Run | 11 Sep 2020 | 36.1 |
| Swartkops | Swartkops 2 | Run | 15 Jun 2021 | 20.2 |
| Swartkops | Swartkops 2 | Run | 5 Feb 2021 | 50.0 |
| Swartkops | Swartkops 3 | Pool | 15 Jun 2021 | 23.5 |
| Swartkops | Swartkops 3 | Pool | 5 Feb 2021 | 40.0 |
| Swartkops | Swartkops 3 | Run | 15 Jun 2021 | 29.5 |
| Swartkops | Swartkops 3 | Run | 5 Feb 2021 | 29.3 |
| Swartkops | Swartkops 4 | Pool | 10 Sep 2020 | 10.4 |
| Swartkops | Swartkops 4 | Pool | 15 Jun 2021 | 16.7 |
| Swartkops | Swartkops 4 | Run | 10 Sep 2020 | 11.6 |
| Swartkops | Swartkops 4 | Run | 15 Jun 2021 | 5.3 |
| Swartkops | Swartkops 5 | Pool | 15 Jun 2021 | 20.0 |
| Swartkops | Swartkops 5 | Pool | 4 Feb 2021 | 25.4 |
| Swartkops | Swartkops 5 | Run | 11 Sep 2020 | 20.2 |
| Swartkops | Swartkops 5 | Run | 15 Jun 2021 | 20.7 |
| Swartkops | Swartkops 5 | Run | 4 Feb 2021 | 16.0 |
| Swartkops | Swartkops 6 | Pool | 14 Jun 2021 | 18.9 |
| Swartkops | Swartkops 6 | Pool | 4 Feb 2021 | 87.5 |
| Swartkops | Swartkops 6 | Run | 14 Jun 2021 | 23.4 |
| Swartkops | Swartkops 6 | Run | 4 Feb 2021 | 20.0 |
| Swartkops | Swartkops 7 | Pool | 14 Jun 2021 | 6.4 |
| Swartkops | Swartkops 7 | Pool | 4 Feb 2021 | 38.5 |
| Swartkops | Swartkops 7 | Pool | 9 Nov 2020 | 45.9 |
| Swartkops | Swartkops 7 | Run | 14 Jun 2021 | 2.7 |
| Swartkops | Swartkops 7 | Run | 4 Feb 2021 | 13.8 |
| Swartkops | Swartkops 7 | Run | 9 Nov 2020 | 23.4 |
| Swartkops | Swartkops 8 | Pool | 14 Jun 2021 | 8.7 |
| Swartkops | Swartkops 8 | Pool | 4 Feb 2021 | 28.8 |
| Swartkops | Swartkops 8 | Run | 14 Jun 2021 | 19.1 |
| Swartkops | Swartkops 8 | Run | 4 Feb 2021 | 14.0 |
| Buffalo | Buffalo Pass | Pool | 11 Feb 2021 | 21.4 |
| Buffalo | Buffalo Pass | Run | 11 Feb 2021 | 12.7 |
| Buffalo | Buffalo Pass | Run | 22 Jun 2021 | 6.8 |

| River | Site name | Hydraulic conditions | Date | Particles $L^{-1}$ |
|---|---|---|---|---|
| Buffalo | Eluphonelweni | Pool | 11 Feb 2021 | 42.9 |
| Buffalo | Eluphonelweni | Pool | 23 Jun 2021 | 44.3 |
| Buffalo | Eluphonelweni | Run | 11 Feb 2022 | 39.1 |
| Buffalo | Eluphonelweni | Run | 23 Jun 2021 | 26.7 |
| Buffalo | Postdam | Pool | 22 Jun 2021 | 25.0 |
| Buffalo | Postdam | Run | 22 Jun 2021 | 19.2 |
| Buffalo | River road | Pool | 11 Feb 2021 | 16.9 |
| Buffalo | River road | Run | 11 Feb 2021 | 9.7 |
| Buffalo | Zwelitsha bridge | Pool | 11 Feb 2021 | 14.2 |
| Buffalo | Zwelitsha bridge | Run | 11 Feb 2021 | 18.5 |
| Buffalo | Zwelitsha bridge | Run | 22 Jun 2021 | 6.7 |